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CONTRACTING ORGANIZATION: Henry M. Jackson Foundation for the Advancement of Military Medicine, Rockville, Maryland 20852

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13. ABSTRACT *(Maximum 200 words)*

Taxol is a microtubule poison that has been used successfully in refractory breast cancer. Apart from its well characterized anti-mitotic effects, Taxol shares with bacterial lipopolysaccharide (LPS) the capacity to elicit microtubule-independent, intracellular signaling in murine macrophages leading to expression of many genes. This IDEA grant proposed to test the ability of Taxol to up-regulate expression of two genes, *nm23* and *c-kit*, whose expression is down-regulated in advanced, metatstatic breast cancer. Modulation of adrenomedulin (AM), as well as a panel of inflammatory genes, were also examined in murine macrophages and/or breast cancer cells stimulated by LPS or Taxol. Using optimized conditions for detection of mRNA species by reverse transcriptase polymerase chain reaction (RT-PCR), we (1) completed studies on the modulation of AM in in macrophages, (2) demonstrated differential modulation of NM-23 and c-kit mRNA in in the murine breast cancer cell line, DA-3, and (3) demonstrated that LPS and/or Taxol strongly up-regulate expression of a panel pro-inflammatory genes in a murine (DA-3) and a human (MDA-MB-231) breast cancer cell lines. (4) Lastly, in the presence of DA-3 tumor cells, stimulation of macrophages by LPS or Taxol plus IFN-γ results is a significant increase in the capacity of the macrophages to release nitric oxide (NO), shown previously to be tumoricidal. *In toto*, these studies largely complete and exceed the proposed scope of the research outlined in our original proposal.

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FOREWORD

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X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Stephanie Vogel 7/20/99

PI - Signature Date

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INTRODUCTION

Taxol™ (generically referred to as paclitaxel) is the prototype of a new class of microtubule stabilizing agents that has generated great enthusiasm in the oncology community due to its favorable response rate in patients with aggressive, metastatic cancers, including breast cancer (1). In addition to its anti-mitotic effects on cells, which is mediated by its ability to bind to β-tubulin and prevent the microtubule depolymerization required for cell division, Taxol has also been shown in murine macrophages to mimic the action of bacterial lipopolysaccharide (LPS) to modulate gene expression and tyrosine phosphorylation of proteins (2). More recently, Haskill *et al.* (3) have found that Taxol also modulates inflammatory gene expression in certain primary ovarian tumor cells. For this reason, we hypothesized that Taxol may also exert its anti-tumor effects by modulating the expression of certain genes whose expression is dysregulated in breast cancer. We proposed in our original grant to analyze in both macrophages and breast cancer cells the effects of Taxol and LPS on the expression of two genes, *nm23* and *c-kit*, whose expression has been found to be down-regulated in breast cancer (4,5). Conversely, the gene that encodes adrenomedullin (AM) has been reported to be expressed by many cancer cells and AM has recently been implicated as an autocrine growth factor in malignant cells (6). For this reason, expression of the AM gene, and its modulation by Taxol and LPS, were also analyzed in macrophages and breast cancer cells. The main hypothesis to be tested is that Taxol and/or LPS may modulate expression of these genes not only in macrophages, but also in in breast cancer cells, where their expression is dysregulated. Such a modulation could result in restoration of normal homeostatic gene expression or the production of pro-inflammatory genes by the tumor that activate immune cell types, such as macrophages, to attack the tumor.

BODY

In our first progress report (7/97), we detailed our findings with respect to the (1) development of the capability to detect NM-23, c-kit, and AM mRNA expression by RT-PCR technology, (2) confirmation of detection of these species in murine melanoma cell lines and murine macrophages (the RAW 264.7 or C3H/OuJ macrophages), (3) the failure of either LPS or Taxol to modulate NM-23 or c-kit significantly, under conditions where AM mRNA was strongly upregulated. Since submitting that annual report, a manuscript on the regulation of AM mRNA expression by LPS and Taxol was published in the peer-reviewed journal, *Infection and Immunity*, and a reprint of this paper is now included in the Appendix (Zaks-Zilberman *et al.*, "Induction of Adrenomedullin mRNA and Protein Expression by Lipopolysaccharide and Paclitaxel (Taxol) in Murine Macrophages"). These data were presented, in part, at the NIH Adrenomedullin Symposium that was held in September 1997.

In the second year of funding (Progress Rept. dated 7/98) and during this final no-cost extension year, we continued our studies, focusing first on an analysis of gene expression in the murine breast cancer cell line, DA-3, and then later in the human

breast cancer cell line, MDA-MB-231. We failed to observe significant modulation of NM-23 and c-kit mRNA by LPS or Taxol in either the murine macrophages or murine melanoma cell lines, in contrast to the strong modulation of AM expression detected in macrophages. However, the recent findings of White *et al.* (7), that Taxol induced expression of the cytokine gene IL-1β, but not the chemokine gene, IL-8, in a murine breast cancer cell line, MCF-7, led us to expand our panel of candidate genes to include a wide variety of cytokine (*e.g.*, IL-1β, GM-CSF, M-CSF, G-CSF, IL-12 p40), chemokine (*e.g.*, IP-10, MIP-2, JE, MIP-1β, MCP-5), and other (*e.g.*, iNOS) genes, that we had found in our previous studies to be inducible by both LPS and Taxol in murine macrophages. In general, the cytokines chosen for this analysis have been strongly implicated in the induction and amplification of inflammatory responses, and the chemokines, for their capacities to cause the influx of inflammatory cell types into the affected region. We began our analysis of the murine DA-3 cell line by treating it with medium only, LPS (100 ng/ml), or paclitaxel (35 μM), concentrations found to induce maximal levels of mRNA in murine macrophages. Total RNA was isolated and levels of steady-state mRNA expression were assessed by RT-PCR. The conditions established for RT-PCR depended upon the identification of primer sets and probes for Southern blotting, as well as optimization of cycle number for gene detection (Table 1). Table 2 lists those genes that were not detected in RNA derived from the DA-3 cells over an 8 h timecourse, even after 40 cycles of PCR amplification, without or with treatment (including c-kit). Of the genes for which basal mRNA levels could be detected, NM-23 (A) and M-CSF were only slightly induced (<3-fold) by LPS or Taxol (data not shown). In contrast, moderate to high increases (>3 to 30-fold) in mRNA levels were observed for G-CSF, MIP-2, iNOS, IL-1β, GM-CSF, and IP-10.

The kinetics of those genes induced by LPS and/or Taxol at moderate to high levels was more extensively characterized. In DA-3 cells, the kinetics and magnitude of induction by LPS or paclitaxel of IL-1β, G-CSF, MIP-2, and iNOS was very similar (Figure 1). All four genes were induced within 2 hr of stimulation, and peak levels seen 2-6 h after stimulation. By 24 h after treatment, steady-state mRNA expression of all four genes had begun to decline, although iNOS mRNA expression remained significantly elevated. As has been observed in murine macrophages (23), the similar and often overlapping kinetic profiles of gene induction by paclitaxel and LPS suggest that they operate via a shared signal transduction pathways in the DA-3 breast cancer cell line. The kinetics of mGM-CSF mRNA expression induced by LPS and paclitaxel in the murine DA-3 cell line were also similar, peaking by 2 h, and returning rapidly to basal levels by 24 h (Fig. 2; top left panel). The peak magnitude induced by LPS was consistently one of the highest elicited among all fourteen genes in our panel. In contrast, the expression of the IP-10 gene was induced much more strongly in DA-3 cells by Taxol than by LPS, resulting in a maximum induction of ~30-fold above basal levels at 24 h following stimulation with Taxol (Fig. 2; bottom left panel). To assess the sensitivity of the DA-3 cell line to induction of mGM-CSF by LPS or Taxol, dose response analyses were also performed (Figure 3). The DA-3 cell line was treated for 2 h, the time when mGM-CSF mRNA expression had peaked, with various concentrations of LPS or Taxol. As little as 0.01 ng/ml LPS induced a detectable increase in GM-CSF mRNA expression, while higher concentrations were necessary

relationships establish the sensitivity of the murine DA-3 cell line to be comparable to that observed in murine macrophages treated with LPS or paclitaxel (9).

We also analyzed expression of GM-CSF and IP-10 genes in a human breast cancer cell line, MDA-MB-231, which, like the murine DA-3 line, has been shown previously to express constitutive levels of GM-CSF (10). However, LPS, but not paclitaxel, induced expression of human (h) GM-CSF mRNA in the human MDA-MB-231 cell line (Fig. 2; top right panel). In contrast, both agents strongly induced hIP-10 gene expression, peaking at 2 and 8 h following LPS stimulation and paclitaxel stimulation, respectively, with levels remaining elevated (~20-30-fold) for >24 h (Fig. 2; bottom right panel). At the level of protein, the human MDA-MB-231 cell line was stimulated by LPS to secrete ~10-fold more hGM-CSF than media- or paclitaxel-treated cells (Fig. 4). Similar results were observed for the production of hIP-10, where medium- and paclitaxel-treated MDA-MB-231 cells secreted hIP-10 below the level of detection (100 pg/ml), whereas LPS-stimulated MDA-MB-231 cells produced 4,946 $\pm$ 350 pg/ml hIP-10. Due to the low sensitivity of the murine IP-10 ELISA, we could not determine if IP-10 protein was produced by LPS- or Taxol-stimulated DA-3 cells.

We have previously reported that LPS and Taxol synergize with IFN-γ to induce nitric oxide (NO) release by LPS-responsive (C3H/OuJ) macrophages, but not in LPS-unresponsive (C3H/HeJ) macrophages (8) and that *in vitro* tumoricidal activity induced by IFN-g plus either LPS or Taxol was NO-dependent (8). To determine if the presence of tumor cells modulates macrophage NO production, murine breast cancer cells and macrophages were co-cultured and treated with medium only, IFN-γ (5 U/ml), LPS (100 ng/ml), paclitaxel (35 μM), or the combination of stimuli. Fig. 5 shows that neither DA-3 breast cancer cells nor C3H/HeJ macrophages responded to any stimuli to release NO. The failure of C3H/HeJ macrophages to release NO was not reversed by the presence of DA-3 breast cancer cells and LPS/paclitaxel and IFN-γ. However, C3H/OuJ macrophages stimulated by IFN-γ plus either either LPS or paclitaxel secreted significantly more NO than macrophages stimulated with either stimulant alone, confirming our previous observation of synergistic induction of iNOS and NO release in these macrophages (8). The concurrent presence of the DA-3 cells in the C3H/OuJ macrophage cultures resulted in a significant increased in macrophage secretion of NO in response to paclitaxel or LPS plus IFN-γ. We are very close to submitting this work for consideration for publication in a peer-reviewed journal and our most recent draft is included in the Appendix (Zaks-Zilberman *et al.*, manuscript in preparation).

The mechanisms by which LPS and Taxol act on tumor cells to induce gene expression and protein expression remain to be delineated, although this is well beyond the scope of the proposed project. However, using Western blot analysis, we were able to determine that two major LPS receptors, CD14 and Mac-1, are not detectable on the DA-3 breast cancer cell line. Moreover, Toll-like receptors, TLR2 and TLR4, have been recently implicated in LPS and Taxol signaling in murine macrophages. The DA-3 cell line was found to express constitutive levels of TLR2 and TLR4 mRNA, suggesting the possibility that these two receptors may mediate

signaling observed in response to LPS or Taxol. Finally, an inhibitor of both LPS and Taxol, *Rhodobacter sphaeroides* diphosphoryl lipid A, blocked induction of GM-CSF gene expression in the DA-3 cell line (data not shown).

CONCLUSIONS

During the first year of funding on this project, we developed all of the molecular methodology required to evaluate the effects of Taxol and LPS on the specific expression of NM-23, c-kit, and AM mRNA in murine macrophages and in breast cancer cell lines. In addition, differential expression of the three genes in control melanoma cells and in the macrophages was observed. Taxol and LPS up-regulated expression of AM, and to a lesser extent, c-kit and NM-23, in macrophage cultures. We completed our analysis of the effects in macrophages of LPS and Taxol on genes that have been reported in the literature to be either repressed (e.g., NM-23 or c-kit) or augmented (e.g., AM) in breast cancer cells. Thus, the finding that neither NM-23 nor c-kit expression were markedly altered under conditions of either LPS or Taxol stimulation indicates that our initial hypothesis is not acceptable.

During the second year of funding, we continued our work as proposed initially, by extending our analysis of gene expression in murine macrophages and a melanoma cell line to an analysis of these genes, as well as others, in the murine breast cancer cell line, DA-3. Although we were unable to demonstrate modulation of c-kit mRNA in these cells, NM-23 mRNA was induced by both LPS and Taxol, albeit to a relatively low extent. In recent years, we and others have shown that in murine macrophages, Taxol induces the expression of a number of genes and secretion of many inflammatory gene products, via a signal transduction pathway that is indistinguishable from that of LPS (reviewed in 11). Since many of the cytokine genes induced by LPS and Taxol can mediate tumor regression, the possibility that this gene-inducing activity is responsible for at least some of the anti-tumor activity seen *in vivo* remains a viable hypothesis. Our data generated in the second and third years of this project strongly support the exciting hypothesis that Taxol and LPS induce in a murine breast cancer cell line a wide array of pro-inflammatory cytokine genes, as well as genes that encode chemokines, factors that are capable of attracting inflammatory cell types to the tumor site. The finding that the human breast cancer cell line, MDA-MB-231 also exhibits increased steady-state levels of mRNA expression of the cytokine, GM-CSF, in response to LPS, and of the chemokine, IP-10, in response to LPS or Taxol, further supports these findings. Thus, it is likely that the observed efficacy of Taxol, as opposed to other agents (*e.g.*, vinblastine or colchicine) that also target microtubules to elicit their anti-tumor actions, may be attributable to Taxol's additional ability to mount an inflammatory response against the tumor by causing the tumor to produce proinflammatory cytokines and chemokines that, in turn, recruit and activate inflammatory cell types, such as macrophages. Lastly, we have provided evidence that the presence of tumor cells increases the capacity of macrophages to respond to potent activation stimuli, *e.g.*, IFN-γ plus either LPS or Taxol, to release NO. This final no-cost extension year has enabled us to complete the tasks proposed in the initial proposal.

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APPENDICES:

Tables 1, 2

Figure legends

Figures 1-5

BIBLIOGRAPHY:

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Zaks-Zilberman, M., Salkowski, C.A., Elsasser, T., Cuttitta, F., and Vogel, S.N. 1998. Induction of adrenomedullin mRNA and protein by lipopolysaccharide and paclitaxel (Taxol) in murine macrophages. *Infect. Immun.*, 66: 4669-4675.

Zaks-Zilberman, M., Zaks, T. Z., and Vogel, S. N. 1999. Induction of proinflammatory and chemokine genes by lipopolysaccharide and paclitaxel (Taxol™) in murine and human breast cancer cell lines. (*in preparation*).

ADDITIONAL NOTES:

Meirav Zaks-Zilberman was also a registered attendee at the first Department of Defense (DOD) Breast Cancer Research Program Era of Hope Meeting, Washington, DC.

Meirav Zaks-Zilberman was the only person whose salary was derived from this IDEA grant.

Table 1. Conditions for gene expression analysis by reverse transcriptase polymerase chain reaction (RT-PCR)

Species	Gene	Annealing Temp [°C]	Cycles amplified[a]
Murine (m)	GM-CSF	54	28
	G-CSF	57	26
	NM 23 (A)	50	25
	M-CSF	58	28
	IP-10	55	31
	MIP-1β	65	40
	MIP-2	65	33
	iNOS	65	32
	IL-1β	54	32
	IL-12 p40	58	40
	MCP-5	55	40
	c-kit	53	40
	AM	55	40
	JE/MCP-1	65	40
	HPRT	54	24
Human (h)	GM-CSF	56	27
	IP-10	56	33
	β-actin	53	24

[a] The number of cycles used for the detection of each gene under nonsaturating conditions.

Table 2. LPS- or paclitaxel-induced mRNA expression in mouse DA-3 breast cancer cells

Level of mRNA induction by LPS or paclitaxel		
Not Detected **Not Induced[a]**	**Low Induction** **(<3-fold)**	**Moderate to High induction** **(>3-fold)**
IL-12 p40	NM 23 (A) (4)[b]	G-CSF (4)
MIP-1β	M-CSF (2)	MIP-2 (2)
MCP-5		iNOS (4)
c-kit		IL-1β (4)
AM		GM-CSF (5)
JE/MCP-1		IP-10 (4)

[a]mRNA expression was undetectable with any treatment regimen after 40 cycles of PCR.

[b]Numbers in parentheses indicate the number of independent experiments.

FIGURE LEGENDS

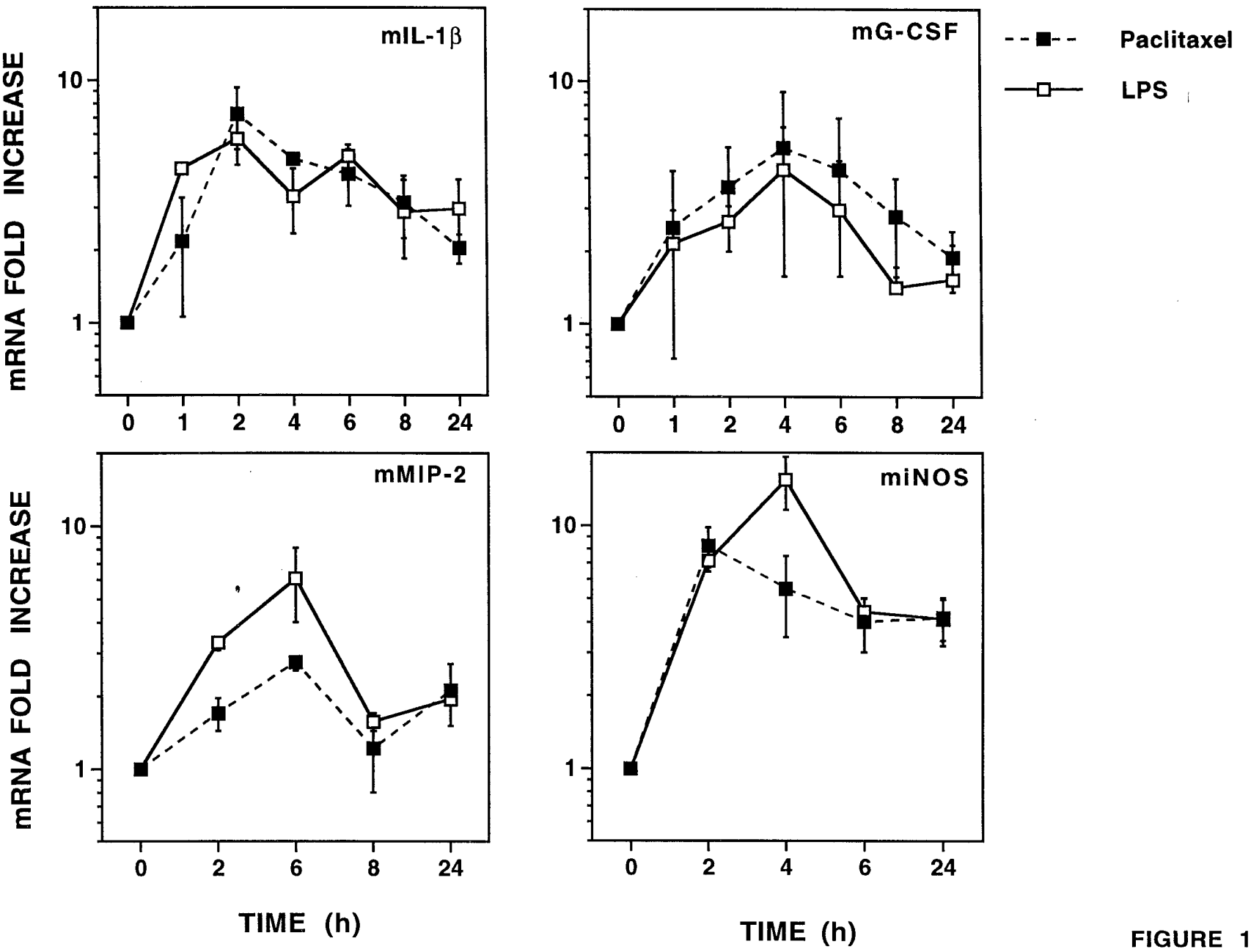
Figure 1. IL-1ß, G-CSF, MIP-2, and iNOS mRNA are induced in the DA-3 murine breast cancer cell line by both paclitaxel and LPS. Cells were cultured for the indicated time with medium, 100 ng/ml LPS, or 35 μM paclitaxel, and levels of mRNA for IL-1β, G-CSF, MIP-2 or iNOS were quantified by RT-PCR. Data represent the arithmetic mean ± s.e.m. from 4 independent experiments.

Figure 2. Induction of GM-CSF and IP-10 mRNA in DA-3 (murine) and MDA-MB-231 (human) breast cancer cell lines. Cell lines were cultured for the indicated time with medium, 100 ng/ml LPS, or 35 μM paclitaxel, and the kinetics of GM-CSF and IP-10 mRNA levels were quantified by RT-PCR. Data represent the arithmetic mean ± s.e.m. from 5 independent experiments.

Figure 3. Dose-dependent induction of GM-CSF mRNA in murine DA-3 cell line. The DA-3 breast cancer cells were cultured for 2 h with medium or with the indicated concentrations of LPS or paclitaxel. Data represent the arithmetic mean ± s.e.m. from 3 independent experiments.

Figure 4. GM-CSF protein secretion induced by LPS or paclitaxel in the human MDA-MB-231 line. Culture supernatants following treatment with 100 ng/ml LPS or 35 μM paclitaxel were collected at the times indicated and assayed for GM-CSF protein content by ELISA. Data are representative of 3 independent experiments with similar results.

Figure 5. Paclitaxel or LPS plus IFN-γ induce augmented macrophage NO release in the presence of breast cancer cells. C3H/OuJ or C3H/HeJ macrophages, DA-3 murine breast cancer cells, or co-culture of C3H/OuJ and DA-3 or C3H/HeJ and DA-3, were stimulated with medium alone, 100 ng/ml LPS, 35 μM paclitaxel, 5 U/ml IFN-γ, or IFN-γ plus either LPS or paclitaxel. After 24 h, culture supernatants were harvested for nitrite levels. Values are the arithmetic means ± s.e.m. obtained from four separate experiments. The asterisk indicates a significant difference in NO release stimulated by paclitaxel or LPS and IFN-γ in the absence vs. present of DA-3 cells.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mMIP-2
miNOS
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mRNA FOLD INCREASE
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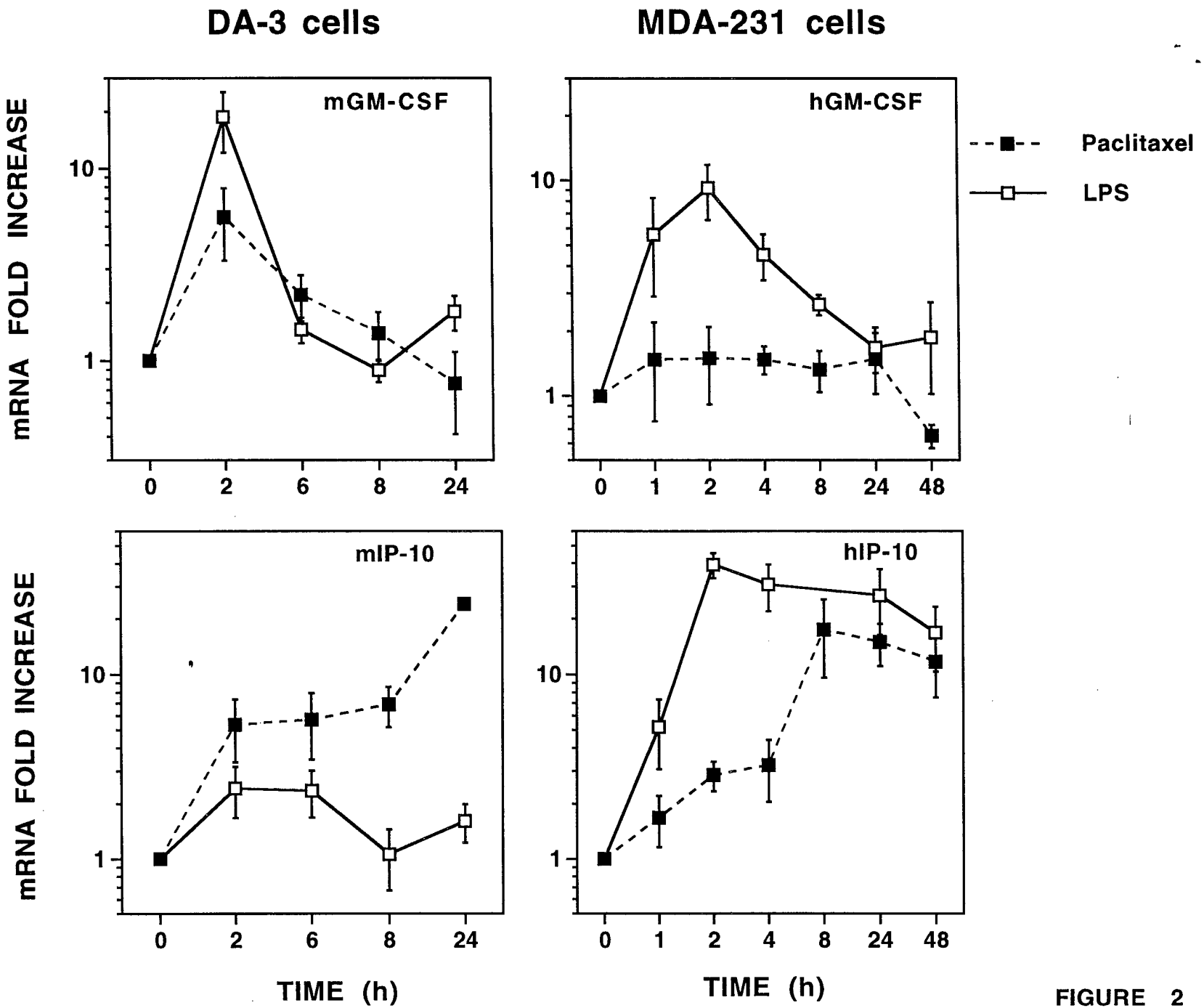
0 2 4 6 24
TIME (h)
TIME (h)
Paclitaxel
LPS

FIGURE 1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MDA-231 cells
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hGM-CSF
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hIP-10
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mRNA FOLD INCREASE
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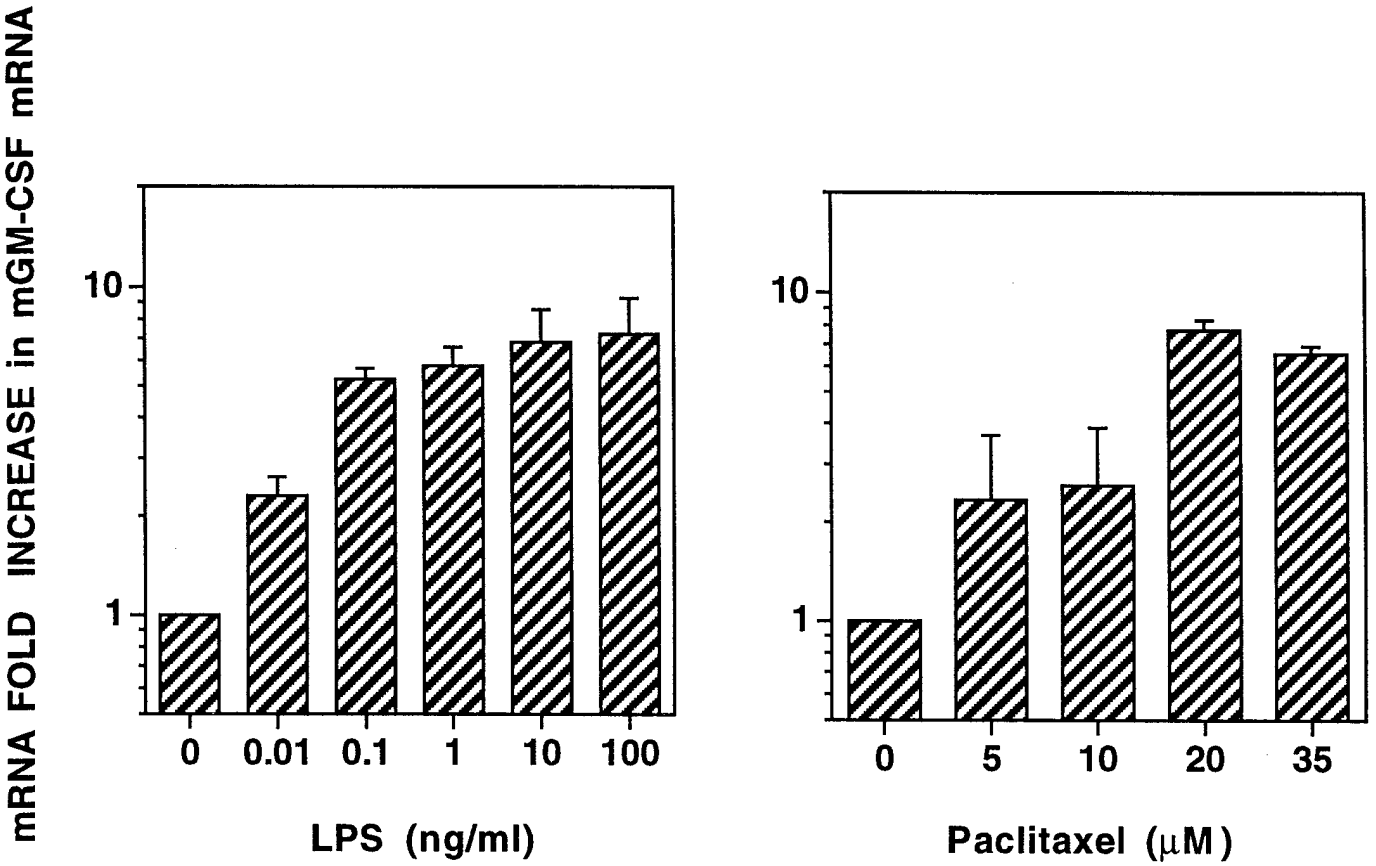
TIME (h)
TIME (h)
Paclitaxel
LPS

FIGURE 2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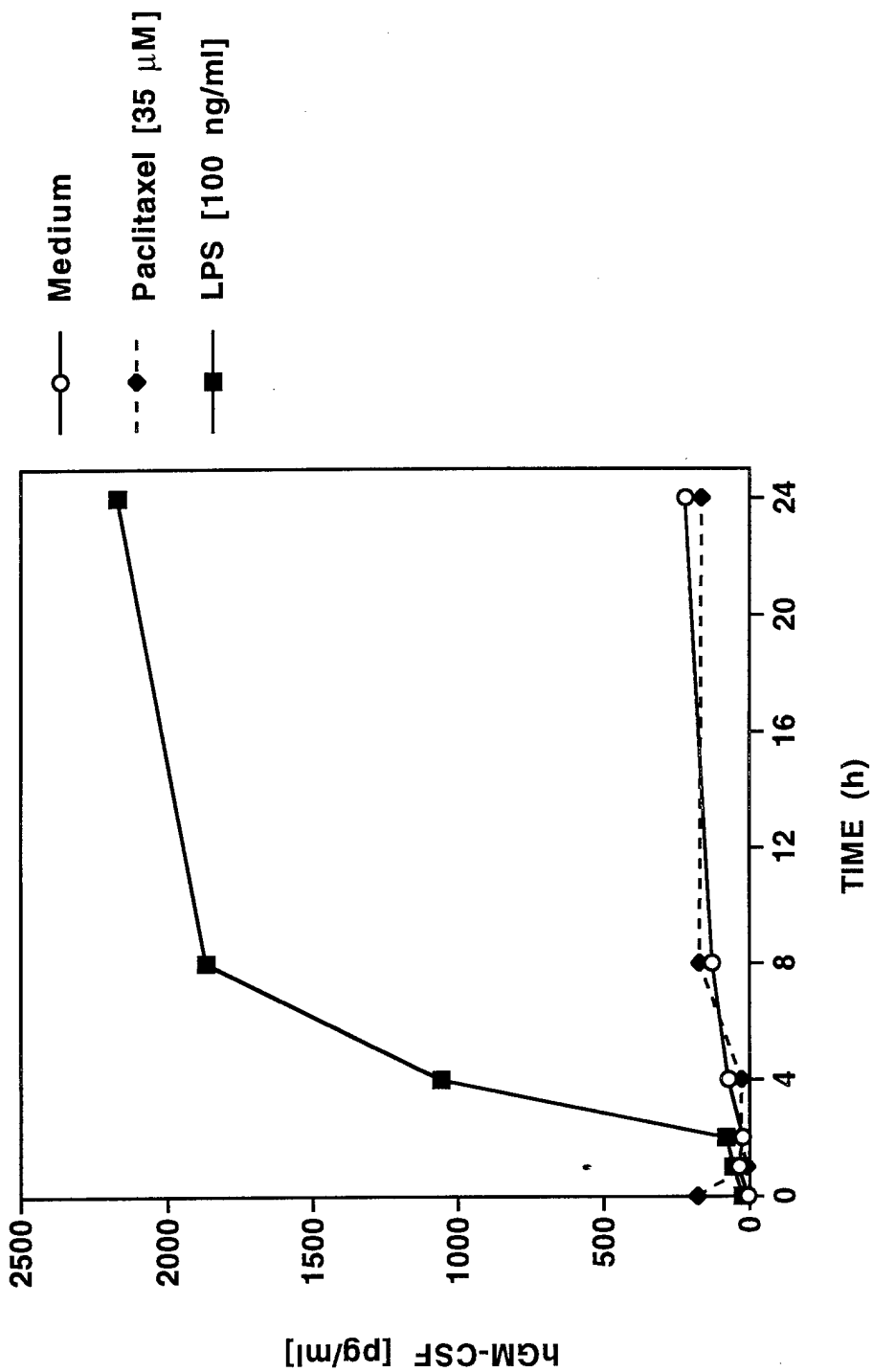
20
35
Paclitaxel (μM)

FIGURE 3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LPS [100 ng/ml]
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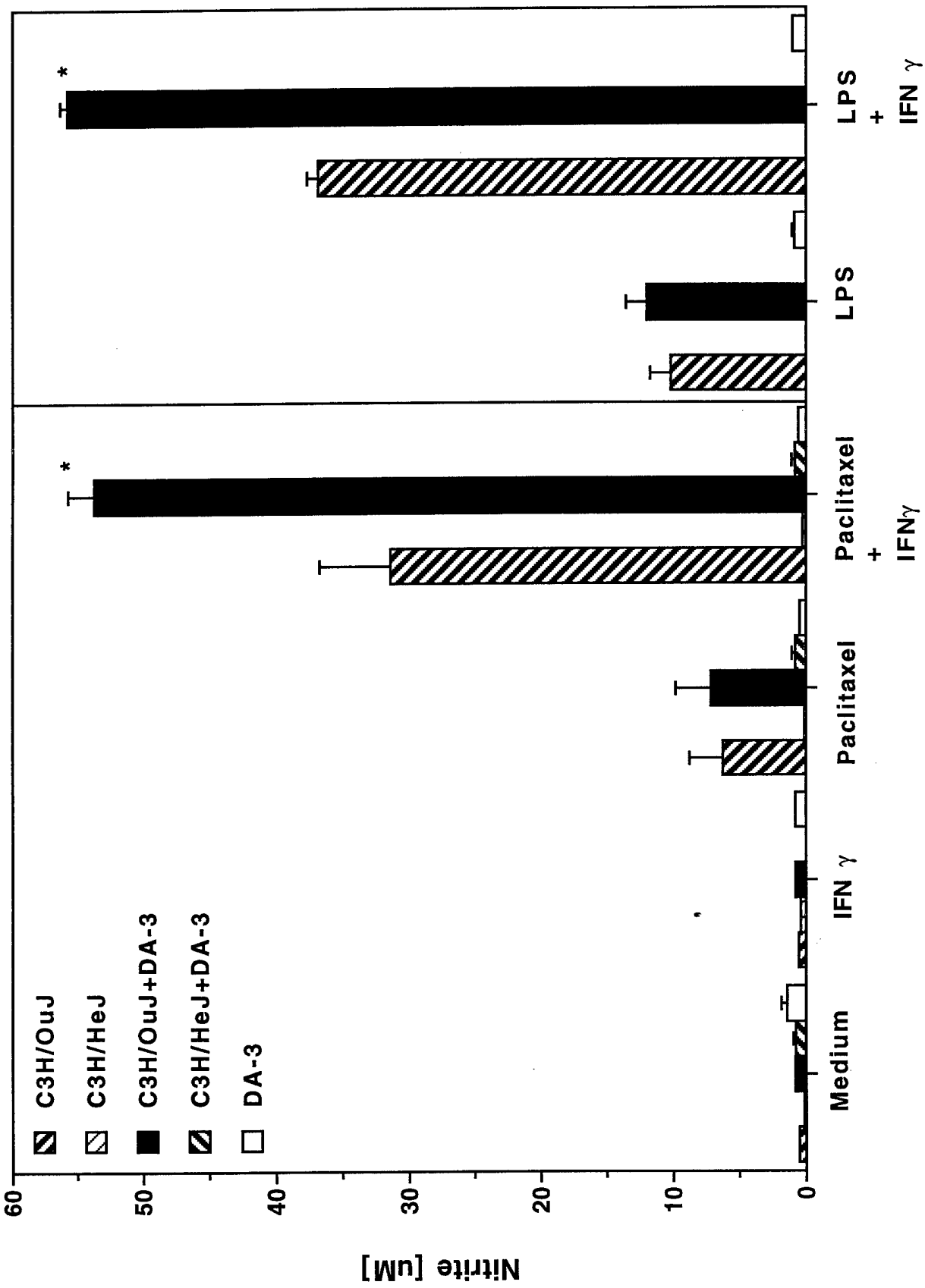
12
16
20
24
TIME (h)

FIGURE 4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50
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30
20
10
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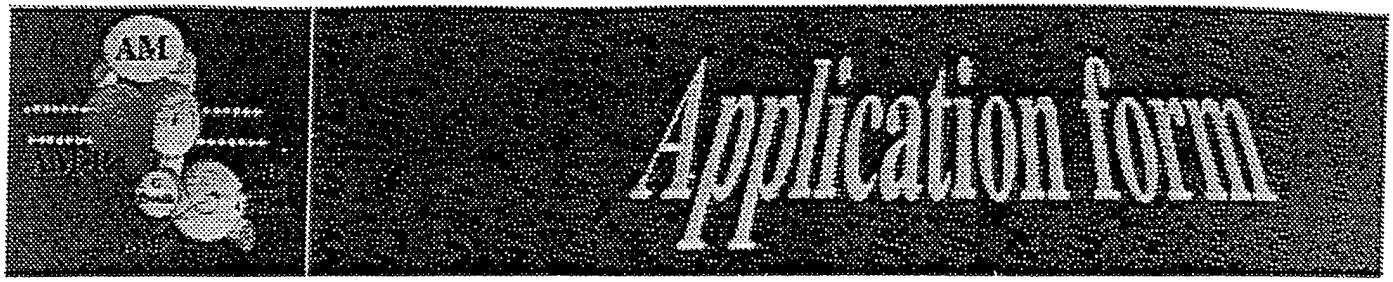
C3H/HeJ
C3H/OuJ+DA-3
C3H/HeJ+DA-3
DA-3
Medium
IFN γ
Paclitaxel
Paclitaxel + IFNγ
LPS
LPS + IFN γ
*
*

FIGURE 5

symposium date: 9/3-5/97
The symposium will take place at the Natcher Conference Center in Bethesda, MD.



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UPREGULATION OF MURINE ADRENOMEDULLIN mRNA EXPRESSION BY LIPOPOLYSACCHARIDE (LPS) AND TAXOL.

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Lipopolysaccharide (LPS) is a potent inflammatory stimulus derived from the outer membrane of Gram negative bacteria that has been implicated in septic shock. LPS stimulation of macrophages leads to a complex series of intracellular signaling events that culminate in the expression of a large number of inflammatory and anti-inflammatory genes. Recently, the antitumor agent, Taxol, was demonstrated to be an LPS-mimetic in murine macrophages. Since adrenomedullin (AM) is a potent vasorelaxant and since septic shock has been associated with vasorelaxation, we investigated the capacity of LPS and Taxol to induce AM in murine macrophages. When peritoneal exudate macrophages from C3H/OuJ mice were treated with protein-free, *E. coli* K235 LPS (100 ng/ml) or Taxol (35 μg/ml), a ~5-fold increase in AM steady-state mRNA levels was observed, peaking between 2 and 4 hours, and returning to baseline after 8 hours. While LPS-hyporesponsive C3H/HeJ macrophages failed to respond to protein-free LPS with an increase in AM steady-state mRNA levels, increased levels were observed after stimulation of these cells with a protein-rich (butanol-extracted) LPS preparation. In addition, increased AM mRNA was observed following treatment of either C3H/OuJ or C3H/HeJ macrophages with soluble *T.gondii* tachyzoite antigen (STAg) or the synthetic flavone analogue 5,6-dimethylxanthenone -4- acetic acid (5,6-MeXAA). Interferon-γ also stimulated C3H/OuJ macrophages to express increased AM mRNA levels. *In vivo*, LPS administration also led to augmented AM gene expression. Mice challenged with 25 μg LPS (i.p.) exhibited increased AM mRNA levels in lung, liver, and spleen, with the greatest increase (>50-fold) observed in the liver. These data indicate that AM represents yet an additional LPS-inducible gene product that may contribute to the induction of Gram negative septic shock through its action on the vascular system. (Supported by NIH AI-18797 and USAMRDC DAMD17-96-1-6258.)

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Induction of Adrenomedullin mRNA and Protein by Lipopolysaccharide and Paclitaxel (Taxol) in Murine Macrophages

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Lipopolysaccharide (LPS), a potent inflammatory stimulus derived from the outer membrane of gram-negative bacteria, has been implicated in septic shock. Plasma levels of adrenomedullin (AM), a potent vasorelaxant, are increased in septic shock and possibly contribute to the characteristic hypotension. As macrophages play a central role in the host response to LPS, we studied AM production by LPS-stimulated macrophages. When peritoneal exudate macrophages from C3H/OuJ mice were treated with protein-free LPS (100 ng/ml) or the LPS mimetic paclitaxel (Taxol; 35 μM), an ~10-fold increase in steady-state AM mRNA levels was observed, which peaked between 2 and 4 h. A three- to fourfold maximum increase in the levels of immunoreactive AM protein was detected after 6 to 8 h of stimulation. While LPS-hyporesponsive C3H/HeJ macrophages failed to respond to protein-free LPS with an increase in steady-state AM mRNA levels, increased levels were observed after stimulation of these cells with a protein-rich (butanol-extracted) LPS preparation. In addition, increased AM mRNA was observed following treatment of either C3H/OuJ or C3H/HeJ macrophages with soluble *Toxoplasma gondii* tachyzoite antigen or the synthetic flavone analog 5,6-dimethylxanthenone-4-acetic acid. Gamma interferon also stimulated C3H/OuJ macrophages to express increased AM mRNA levels yet was inhibitory in the presence of LPS or paclitaxel. In vivo, mice challenged intraperitoneally with 25 μg of LPS exhibited increased AM mRNA levels in the lungs, liver, and spleen; the greatest increase (>50-fold) was observed in the liver and lungs. Thus, AM is produced, by murine macrophages, and furthermore, LPS induces AM mRNA in vivo in a number of tissues. These data support a possible role for AM in the pathophysiology of sepsis and septic shock.

Lipopolysaccharide (LPS) is a potent inflammatory stimulus derived from the outer membrane of gram-negative bacteria. Release of LPS from dying bacteria can initiate a serious systemic inflammatory response to infection, resulting in septic shock. Septic shock is typified by fever, hypoglycemia, hypotension, disseminated intravascular coagulation, multiorgan failure, and shock that may result in death (5, 33, 34). Septic shock continues to have an associated mortality rate of 40 to 70% and remains the leading cause of death in intensive care units (1, 33, 34). The interaction of LPS with host cells initiates the production of a cascade of proinflammatory mediators that are responsible for its effects (25). The release of cytokines like tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-12, interferon-γ (IFN-γ), nitric oxide ($NO^{\cdot}$), and colony-stimulating factor from monocytes and macrophages elicits the physiologic changes observed during sepsis and septic shock (25, 30, 34, 40). The antitumor agent paclitaxel (Taxol) is an LPS mimetic in murine macrophages. Shared activities include the ability to activate murine macrophages to express a wide variety of inflammatory and anti-inflammatory genes, tyrosine phosphorylate mitogen-activated protein kinases (MAPKs), secrete cytokines, induce translocation of NF-κB, and upregulate autophosphorylation of *Lyn* kinase. In addition, paclitaxel provides a second signal to IFN-γ-primed murine macrophages to become tumoricidal and to produce $NO^{\cdot}$ (8, 11, 22, 24, 36). Macrophage responsiveness to both LPS and paclitaxel is linked to the *Lps* gene. The C3H/HeJ mouse strain expresses a defective allele at this locus, and macrophages derived from this mouse strain are hyporesponsive not only to LPS (45) but also to paclitaxel (22, 24).

Adrenomedullin (AM) is a hypotension-causing peptide that was originally isolated from human pheochromocytoma cells (19). It induces vasorelaxation that leads to a persistent depression of blood pressure (15). In previous studies, AM mRNA was found to be expressed in various organs, including the cardiovascular system, lungs, adrenal glands, cultured endothelial cells, vascular smooth muscle cells, alveolar and endometrial macrophages, and virtually all of the tumor cell lines examined (19, 27, 29, 38, 43, 44, 48). Moreover, AM was recently demonstrated to exhibit direct antimicrobial activity (46). The concentration of AM in plasma is increased in patients with hypertension, septic shock, and heart failure, suggesting that AM may participate in the regulation of blood pressure and contribute to refractory hypotension in septic shock (14, 18). Given the plethora of bioactive peptides released by LPS-activated macrophages, we postulated that AM may also be produced by macrophages in response to LPS as a result of gram-negative infection and, perhaps, contribute to the hypotension associated with gram-negative sepsis and septic shock. In the present study, we demonstrated that LPS and paclitaxel, as well as other potent macrophage stimuli, induce

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AM mRNA and protein expression in murine peritoneal macrophages. Additionally, AM mRNA levels were upregulated in the lungs, liver, and spleen following LPS injection.

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MATERIALS AND METHODS

Reagents. Phenol-water-extracted *Escherichia coli* K235 LPS (PW-LPS; <0.008% protein) was prepared by the method of McIntire et al. (28). Protein-rich, butanol-extracted *E. coli* K235 LPS (But-LPS; ~18% protein) was prepared as described by Morrison and Leive (31). Paclitaxel was kindly provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., and was stored at −70°C as a 20 mM stock solution in dimethyl sulfoxide. A 1 mM stock of paclitaxel contained <0.03 endotoxin U/ml by the *Limulus* amoebocyte lysate assay. 5,6-Dimethylxanthenone-4-acetic acid (5,6-MeXAA) was synthesized by the Cancer Research Laboratory, University of Auckland, Auckland, New Zealand (2, 37). A stock solution of 5,6-MeXAA was freshly prepared for each experiment by solubilizing the compound in sterile, endotoxin-free 5% $NaHCO_3$ by vortexing. Once solubilized, the solution was diluted in supplemented RPMI 1640 medium containing 2% fetal calf serum to obtain a 10-mg/ml stock solution that was then diluted to the required concentration for macrophage stimulation. The endotoxin level of the highest concentration of 5,6-MeXAA used in these experiments was <0.0125 ng/ml as detected by the *Limulus* amoebocyte lysate assay. A soluble extract of *Toxoplasma gondii* tachyzoites (STAg) was a gift from Alan Sher, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Recombinant murine IFN-γ (1.3×10^7 U/ml) was kindly provided by Genentech, Inc. (South San Francisco, Calif.). Cycloheximide (CHX) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and used at a final concentration of 5 μg/ml.

Mice. For in vivo analysis of AM gene induction, 6- to 8-week-old C57BL/6J mice were injected intraperitoneally (i.p.) with 25 μg of LPS. Four mice were used per time point per treatment. GKO mice were a gift from Genentech, Inc. (7).

Macrophage isolation and cell culture conditions. Five- to 6-week-old female C3H/OuJ and C3H/HeJ mice were obtained from The Jackson Laboratory (Bar Harbor, Maine), maintained in a laminar-flow facility under 12-h alternating light-dark cycles, and fed standard laboratory chow and acid water ad libitum. Research was conducted in accordance with the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (13a). Mice were injected i.p. with 3 ml of 3% fluid thioglycolate. Four days later, peritoneal exudate cells were extracted by peritoneal lavage. Cells were washed once with and resuspended in RPMI 1640 medium supplemented with 2 mM glutamine, 100-U/ml penicillin, 100-μg/ml streptomycin, 10 mM HEPES, 0.3% sodium bicarbonate, and 2% fetal calf serum and added to six-well tissue culture plates (Falcon, Lincoln Park, N.J.) at $\sim 4.0 \times 10^6$ cells per well in a final volume of 2.0 ml. The plates were incubated at 37°C and 6% CO_2. After a 12-h adherence period, nonadherent cells were washed off and the adherent macrophages were treated with 2.0 ml of medium or medium containing the indicated substances. For detection of AM in culture supernatants, macrophages were cultured at $\sim 6 \times 10^6$ cells per well in six-well tissue culture plates in a total volume of 3.0 ml, and supernatants were collected at the indicated times after stimulation with LPS or paclitaxel.

Isolation of total cellular RNA. For in vitro experiments, culture supernatants were removed and the cells were solubilized in 1 ml of RNA Stat60 (Tel-Test 'B,' Inc., Friendswood, Tex.). For in vivo experiments, the liver, lungs, and spleen were removed from individual mice and frozen at −70°C. Tissues were homogenized in RNA Stat60. Total cellular RNA was extracted from in vitro and in vivo samples in accordance with the manufacturer's instructions and quantified by spectrophotometric analysis.

Analysis of tissue mRNA by RT-PCR. Relative quantities of mRNAs for hypoxanthine-guanine phosphoribosyltransferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and AM were determined by a coupled reverse transcription (RT)-PCR as detailed elsewhere (9). For the AM gene, the following oligonucleotide sequences were used: sense, 5′-AAGAAGTGGAAT AAGTGGGCG; antisense, 5′-ACCAGATCTACCAGCTAACA; probe, 5′-CC CCCTACAAGCCAGCAATCAG.

Primer sequences for the detection of AM mRNA were chosen by analysis of the murine genomic sequence and amplify a 284-bp product. The probe sequence for AM was chosen in conjunction with the published murine cDNA sequences obtained from GenBank. Primer and probe sequences for the HPRT and GAPDH housekeeping genes have been reported (3). The PCR annealing temperatures were 54, 55, and 54°C for HPRT, GAPDH, and AM, respectively. The numbers of PCR cycles were 28 and 35 for in vitro and in vivo AM induction, respectively. The number of PCR cycles for both HPRT and GAPDH was 24. Amplified products were analyzed by electrophoresis, followed by Southern blotting and hybridization with the nonradioactive internal oligonucleotide probe. Chemiluminescence signals were quantified by using a scanning densitometer (Datacopy GS plus; Xerox Imaging Systems, Sunnyvale, Calif.). To determine the magnitude of change in gene expression, cDNA from a sample known to be positive for AM and HPRT or GAPDH was used to generate standard curves by serial twofold dilution of the positive control and simultaneous amplification. The signal of each band in the standard curve was plotted and subjected to linear regression analysis. The equation from this line was used to calculate the fold induction in test samples. Results were normalized for the relative quantity of mRNA by comparison to HPRT or GAPDH. In each in vitro experiment, means are expressed relative to medium controls. In vivo, means are expressed relative to saline-injected controls ($t = 0$), which were assigned a value of 1.

Detection of AM in macrophage culture supernatants. Levels of immunoreactive AM were detected in macrophage culture supernatants by radioimmunoassay (RIA) as described previously (26).

Statistics. Results were analyzed by using Student's t test for comparisons between two groups.

RESULTS

AM mRNA and protein induced by PW-LPS or paclitaxel in murine macrophages. Previous studies have demonstrated that LPS induces AM mRNA and protein expression in cultured rat aortic vascular smooth muscle cells (42) and in cultured endothelial cells (41). In the present study, we investigated if AM mRNA expression and protein secretion are modulated in murine macrophages by PW-LPS or by the LPS mimetic paclitaxel. Endotoxin-responsive C3H/OuJ macrophages were treated for 1, 2, 4, 6, 8, and 24 h with medium alone, 100-ng/ml PW-LPS, or 35 μM paclitaxel. RNA was isolated, and AM and HPRT mRNAs were detected by RT-PCR. As shown in Fig. 1A, the kinetics of AM gene induction by PW-LPS and paclitaxel were remarkably similar, with AM mRNA expression being induced by PW-LPS or paclitaxel as early as 1 h, peaking at 2 h (>10-fold over the baseline), and gradually returning to basal levels by 24 h. To assess the sensitivity of AM mRNA to induction by PW-LPS or paclitaxel, dose-response analyses were performed (Fig. 1B). Murine C3H/OuJ peritoneal macrophages were treated for 2 h, the time when AM mRNA expression had peaked, with various concentrations of PW-LPS or paclitaxel. RNA was isolated, and AM and HPRT mRNAs were detected by RT-PCR. As little as 0.1-ng/ml PW-LPS induced AM mRNA expression (>2-fold), while ≥10-ng/ml PW-LPS was necessary to induce maximal (>10-fold) AM mRNA expression. A comparable increase in AM gene expression was induced by 5 to 35 μM paclitaxel. Macrophage culture supernatants were also analyzed by RIA for the presence of immunoreactive AM. Figure 1C illustrates that both LPS and paclitaxel induce AM secretion several hours after the appearance of AM mRNA, with a maximal induction of three- to fourfold over the basal levels.

PW-LPS- or paclitaxel-induced AM mRNA requires a normal *Lps* gene product. Previous studies in our laboratory have demonstrated the induction of an extensive panel of inflammatory genes (e.g., those for TNF-α, IL-1β, TNF receptor type 2, IFN-inducible protein 10, D3, and D8) by various LPS preparations or paclitaxel in macrophages derived from LPS-responsive (Lps^n) C3H/OuJ mice (22, 45). In contrast, macrophages derived from LPS-hyporesponsive (Lps^d) C3H/HeJ mice failed to express any of the above genes in response to either PW-LPS or paclitaxel. Despite their inability to respond to PW-LPS, C3H/HeJ macrophages are responsive to But-LPS, the product of a milder extraction process in which LPS remains associated with membrane proteins. Moreover, C3H/HeJ and C3H/OuJ macrophages exhibit comparable sensitivity to endotoxin-associated proteins isolated from protein-rich LPS preparations (12) and to STAg. Both protein-rich LPS and STAg result in tyrosine phosphorylation of MAPK and induce a subset of LPS-regulated genes (21). The antitumor agent 5,6-MeXAA is also active on both C3H/HeJ and C3H/OuJ macrophages (35). Therefore, we next investigated whether any of these agents would induce AM gene expression

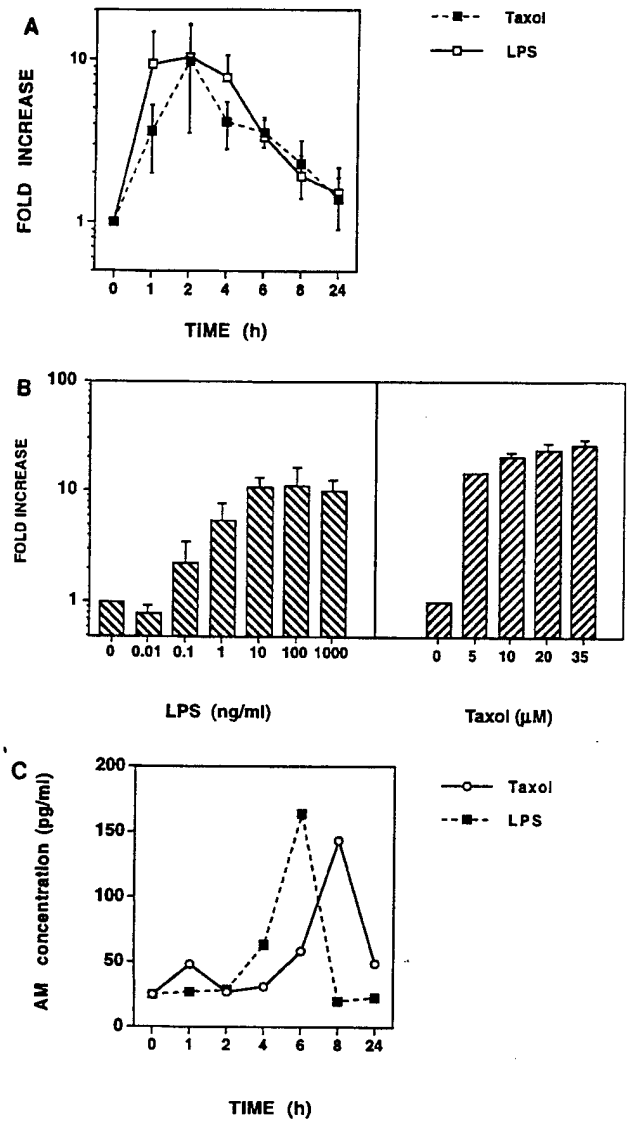

FIG. 1. PW-LPS and paclitaxel induce AM mRNA and protein synthesis in C3H/OuJ macrophages. (A) Kinetics of PW-LPS- and paclitaxel-induced AM mRNA expression. C3H/OuJ macrophages were cultured for the indicated times with medium, 100-ng/ml PW-LPS, or 35 μM paclitaxel. mRNA was isolated, and AM and HPRT mRNAs were detected by RT-PCR. The data represent the arithmetic mean ± the standard error of the mean (seven separate experiments). (B) Dose-dependent induction of AM mRNA. C3H/OuJ macrophages were cultured for 2 h with medium or with the indicated concentrations of PW-LPS or paclitaxel. mRNA was isolated, and AM and HPRT mRNAs were detected by RT-PCR. The data represent the arithmetic mean ± the standard error of the mean (four separate experiments). (C) Kinetics of PW-LPS- and paclitaxel-induced AM secretion. C3H/OuJ macrophages were cultured for the indicated times with medium, 100-ng/ml LPS, or 35 μM paclitaxel. Macrophage culture supernatants were analyzed by RIA for the presence of immunoreactive AM. Data were derived from a representative of three experiments. When not visible, bars indicating the standard error of the mean are smaller than the symbol.

in C3H/HeJ macrophages. Peritoneal macrophages from C3H/HeJ mice were treated with medium alone, 50-μg/ml STAg, 10-μg/ml 5,6-MeXAA, 10-μg/ml But-LPS, 100-ng/ml PW-LPS, or 35 μM paclitaxel for 2 or 4 h. These concentrations were chosen based on optimal induction of gene expression by these agents in previous studies (12, 21, 35). RNA was isolated, and AM and HPRT mRNA levels were quantified. At 2 h, only

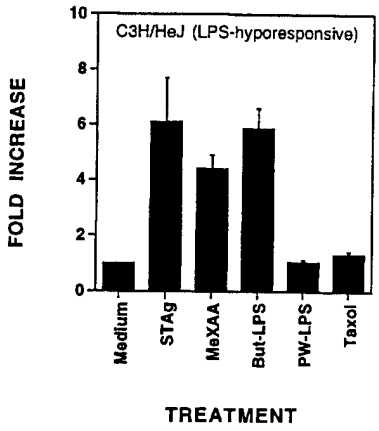

FIG. 2. Neither PW-LPS nor paclitaxel induced AM gene expression in LPS-hyporesponsive C3H/HeJ macrophages in vitro. C3H/HeJ macrophages were treated for 4 h with medium, 5-μg/ml STAg, 10-μg/ml MeXAA, 5-μg/ml But-LPS, 100-ng/ml PW-LPS, or 35 μM paclitaxel. The data represent the arithmetic mean ± the standard error of the mean of four experiments. When not visible, bars indicating the standard error of the mean are smaller than the symbol.

STAg and But-LPS, but not 5,6-MeXAA, had significantly increased AM gene expression (sixfold or more; data not shown). As expected, neither PW-LPS nor paclitaxel induced AM mRNA in C3H/HeJ macrophages (Fig. 2). By 4 h, STAg, But-LPS, and 5,6-MeXAA had increased AM gene expression in C3H/HeJ macrophages greater than four- to sixfold over the baseline (Fig. 2). LPS-responsive macrophages from C3H/OuJ mice also responded to PW-LPS, paclitaxel, STAg, or 5,6-MeXAA by expressing heightened levels of AM mRNA (>10-fold) (data not shown). These data indicate that although the *Lps*^{d} allele precludes induction of AM mRNA by PW-LPS or paclitaxel, these cells respond to STAg, 5,6-MeXAA, and But-LPS with increased expression of AM mRNA.

Expression of AM mRNA in macrophages treated with CHX. To determine whether induction of AM mRNA by LPS or paclitaxel requires de novo protein synthesis, C3H/OuJ macrophages were treated for 2 h with medium, 100-ng/ml PW-LPS, or 35 μM paclitaxel, in the absence or presence of a 5-μg/ml concentration of the protein synthesis inhibitor CHX. This concentration of CHX has been shown previously to inhibit the expression of other LPS-inducible genes in C3H/OuJ macrophages (3). RNA was isolated, and both AM and GAPDH mRNAs were detected by RT-PCR (Fig. 3). CHX alone induced accumulation of steady-state AM mRNA. In

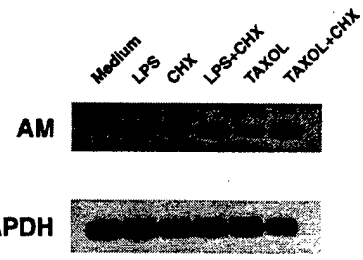

FIG. 3. De novo protein synthesis is not required for PW-LPS- or paclitaxel-induced AM mRNA production. C3H/OuJ macrophages were treated for 2 h with either medium, 100-ng/ml PW-LPS, 5-μg/ml CHX, both 100-ng/ml PW-LPS and 5-μg/ml CHX, 35 μM paclitaxel, or both 35 μM paclitaxel and 5-μg/ml CHX. RNA was isolated, and AM and GAPDH mRNAs were detected by RT-PCR. A representative of three Southern blots is shown.

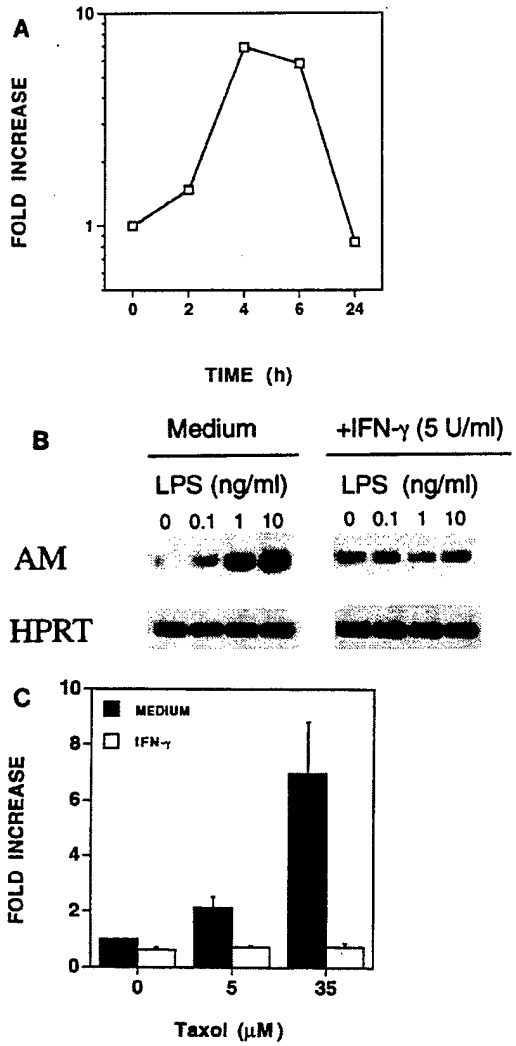

FIG. 4. Regulation of AM mRNA expression by IFN-γ in C3H/OuJ macrophages. (A) IFN-γ upregulates AM mRNA expression. C3H/OuJ macrophages were cultured for the indicated times with medium or 5-U/ml IFN-γ. These data were derived from a representative of two experiments. (B) IFN-γ down-regulates PW-LPS-induced AM mRNA expression. C3H/OuJ macrophages were cultured for 4 h in the presence of medium only or increasing concentrations of PW-LPS in the absence or presence of 5-U/ml IFN-γ. A representative of three Southern blots is shown. (C) IFN-γ down-regulates paclitaxel-induced AM mRNA expression. C3H/OuJ macrophages were cultured for 4 h in the presence of medium only, 5 μM paclitaxel, or 35 μM paclitaxel, in the absence or presence of 5-U/ml IFN-γ. These data represent the arithmetic mean ± the standard error of the mean of three separate experiments.

addition, higher steady-state AM mRNA levels were observed after macrophages were treated for 2 h either with PW-LPS and CHX or with paclitaxel and CHX (Fig. 3). Thus, accumulation of AM mRNA is not dependent on de novo protein synthesis.

IFN-γ upregulates AM mRNA expression and negatively regulates LPS- and paclitaxel-induced AM mRNA expression. We next assessed the ability of a second potent macrophage-activating agent, IFN-γ, to regulate AM mRNA expression. C3H/OuJ peritoneal macrophages were treated with IFN-γ (5 U/ml) for 2, 4, 6, and 24 h. RNA was isolated, and AM and HPRT mRNAs were detected by RT-PCR. As shown in Fig. 4A, AM mRNA expression was induced by IFN-γ as early as 2 h and peaked at 4 to 6 h (greater than sevenfold) and returned to basal levels by 24 h. In many instances, IFN-γ provides a "priming" signal that results in the synergistic induction of gene expression and secreted products (e.g., TNF-α, NO; IL-6) when provided with a second triggering signal such as LPS (13, 47). To ascertain whether IFN-γ would modulate the induction of AM by LPS, C3H/OuJ macrophages were cultured for 4 h with PW-LPS in the absence or presence of IFN-γ. As shown in Fig. 4B, IFN-γ (5 U/ml) down-regulated LPS-induced AM mRNA levels. Similar results were observed when the macrophages were stimulated simultaneously with both paclitaxel and IFN-γ (5 U/ml) (Fig. 4C).

LPS-induced AM mRNA in vivo. Previous studies in this laboratory have demonstrated that LPS elicits gene expression in vivo that is both organ and gene specific (39, 40). To assess whether LPS augments AM mRNA levels in vivo, C57BL/6 mice were challenged i.p. with 25 μg of LPS, and AM mRNA expression was assessed in the liver, lungs, and spleen. As shown in Fig. 5, AM mRNA expression was rapidly induced (by 1 h) in the liver. Hepatic AM mRNA remained at heightened levels (~20- to 60-fold above the baseline) from 3 to 8 h after LPS challenge and then returned to nearly basal levels by 12 h. In contrast, increased AM mRNA expression was not observed in the lungs until 6 to 8 h after LPS challenge, and pulmonary AM mRNA peaked after 12 to 24 h (~50-fold). In contrast to that in both the liver and the lungs, splenic AM mRNA expression was poorly modulated (~4-fold in 3 h) by LPS, and by 24 h, splenic AM mRNA expression was substantially downregulated (~10-fold below basal AM mRNA levels).

Endogenous IFN-γ regulates LPS-induced AM mRNA in vivo. In vitro, IFN-γ suppressed LPS-induced AM mRNA in C3H/OuJ macrophages (Fig. 4B). To examine the role of IFN-γ in the in vivo regulation of AM mRNA by LPS, mice with a targeted disruption in the IFN-γ gene (GKO) (7) were utilized. The basal hepatic AM mRNA level was about fourfold higher in the livers of GKO mice than in those of C57BL/6 mice (Fig. 6). Interestingly, no increase in hepatic AM mRNA was observed after LPS challenge. In fact, by 6 h following LPS administration, AM mRNA levels had returned to the baseline levels exhibited by control C57BL/6 mice.

DISCUSSION

LPS, the endotoxic outer membrane component of gram-negative bacteria, has long been implicated in the pathophysiology of septic shock. The inflammatory syndrome that is associated with sepsis is characterized by hypotension and multiple organ dysfunction, which is felt to be initiated by the action of secondary inflammatory mediators released from LPS-stimulated cells. The systemic inflammatory response induced by LPS or gram-negative bacterial infection can be partially ameliorated by blocking either LPS itself or downstream endogenous mediators, such as IL-1 and TNF-α. Many of these mediators are produced by macrophages. However, in the clinical setting, blocking LPS itself is of limited value since the deleterious effects have already been initiated by the time the inflammatory syndrome is apparent (49). Based on preclinical data, the approach of blocking endogenous inflammatory mediators, such as TNF-α, seemed promising; however, clinical trials have yet to demonstrate the efficacy of using this approach in septic shock (32). AM, originally identified in pheochromocytoma, is a ubiquitously expressed peptide that is a member of the calcitonin-related peptide superfamily (19). It possesses both potent vasodepressor (15, 19) and cardiodepressor (20, 38, 43) activities, and increased plasma AM levels

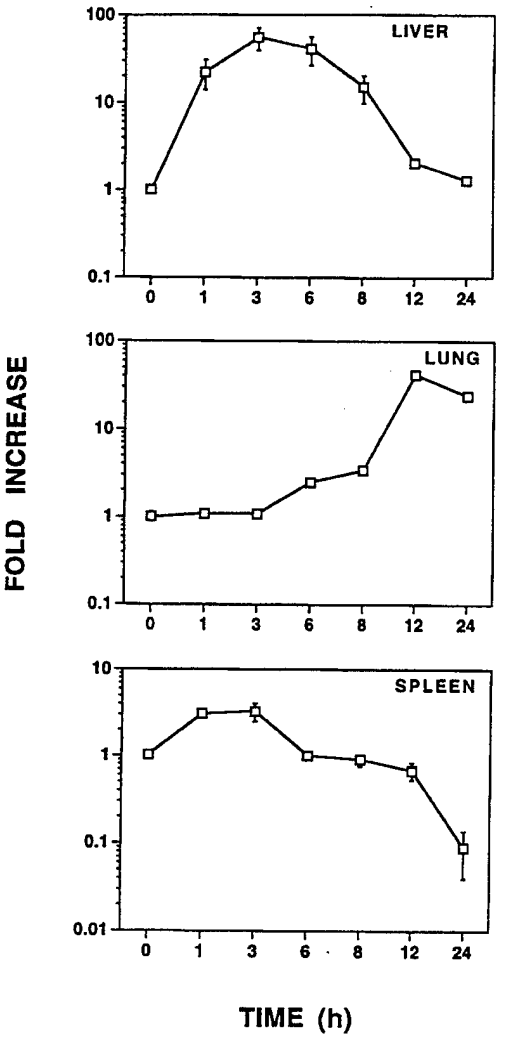

FIG. 5. LPS augments AM mRNA expression in vivo. C57BL/6 mice were injected i.p. with 25 μg of LPS. These data are the mean fold increase in AM mRNA expression ± the standard error of the mean from four to eight individual mice at each time point. Means are expressed relative to that of the saline-injected control (t = 0), which was arbitrarily assigned a value of 1. When not visible, bars indicating the standard error of the mean are smaller than the symbol.

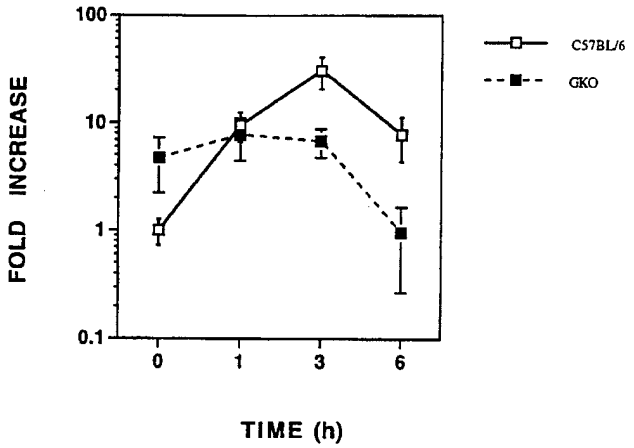

FIG. 6. Endogenous IFN-γ regulates LPS-induced AM mRNA in vivo. GKO and C57BL/6 mice were injected i.p. with 25 μg of LPS, and LPS-induced AM mRNA was quantified in the liver. These data are the arithmetic mean ± the standard error of the mean for five to eight mice at each time point.

have been reported in a variety of clinical conditions associated with blood pressure and hemodynamic alterations, suggesting that it participates in blood pressure regulation (14, 18). While LPS had been shown previously to induce AM gene transcription in endothelial and vascular smooth muscle cells (41), it was not known whether LPS also induces AM in macrophages. The data presented herein demonstrate that LPS causes a rapid induction of AM gene transcription in peritoneal macrophages in vitro, peaking within 2 h and gradually subsiding within 24 h, a kinetic profile very similar to that of other LPS-inducible proinflammatory genes (21, 22, 35). Furthermore, AM gene induction is not dependent upon new protein synthesis, implying the existence of a preformed signal transduction apparatus. Moreover, the finding that CHX alone increased steady-state AM mRNA levels suggests that AM gene expression is maintained in a suppressed state due to the action of a CHX-sensitive suppression molecule. The increase in AM mRNA was followed by secretion of immunoreactive AM into the culture supernatants. Thus, our study lends support to the notion that AM, like TNF-α and IL-1, might serve as an endogenous mediator of the inflammatory syndrome associated with sepsis. This remains to be proven by blocking its action in vivo; however, blocking anti-AM antibodies are not available. More recently, AM has been shown to be directly microbicidal (46), suggesting that AM, like other cytokines and chemokines, can be stimulated by bacterial products, such as LPS, as a normal part of the macrophage's innate response to infection. This hypothesis is strengthened by our findings that two other bacterial stimulants, STAg and endotoxin-associated proteins, were also found to be potent stimuli (Fig. 2).

It is also known that AM significantly enhanced $NO^{\cdot}$ synthesis evoked by LPS and IFN-γ in cultured vascular smooth muscle cells. Thus, AM may contribute to circulatory failure during endotoxin shock, in part, by modulating $NO^{\cdot}$ release (42). However, we were unable to activate C3H/OuJ macrophages with synthetic AM (up to 1 mM) to release $NO^{\cdot}$, and the presence of synthetic AM failed to modulate $NO^{\cdot}$ release stimulated by LPS and/or IFN-γ (data not shown). Thus, it appears that the induction of each of these two vasodilatory substances by LPS is regulated independently.

We have previously shown that the antitumor chemotherapeutic agent paclitaxel mimics the effects of LPS on murine macrophages. Both are dependent upon the expression of a normal *Lps* allele, are blocked by the same LPS analog antagonists, cause tyrosine phosphorylation of MAPKs and autophosphorylation of *Lyn* kinase, induce translocation of NF-κB, and induce an indistinguishable pattern of cytokine gene expression and secretion (6, 25). Furthermore, the effect of paclitaxel appears to be independent of its well-characterized microtubule-binding activity, as evidenced by the failure of paclitaxel analogs with various microtubule-binding capacities to correlate with LPS-mimetic activity (17, 25, 45). The data presented herein demonstrate that, like LPS, AM is induced in murine macrophages by paclitaxel. Interestingly, paclitaxel causes hypotension in ~10% of patients within 3 h of administration, and the mechanism of this side effect is unknown but the data are consistent with the possibility that paclitaxel-induced AM is a contributing endogenous mediator. AM has recently been shown to act as a local autocrine growth factor in

a variety of human tumors (29). The antitumor activity of paclitaxel is believed to be due primarily to its antimitotic activity on tumor cells, although it has also been shown to activate tumoricidal macrophages (4, 24) and to inhibit angiogenesis. The role of paclitaxel-induced production of AM by neoplastic cells will be the focus of future studies.

In vivo, LPS induced an almost 100-fold increase in AM gene expression in the liver, reaching peak expression within a few hours, whereas later induction was observed in the lungs, with fundamentally no AM induction in the spleen. LPS has been shown to cause elevated AM levels in the plasma of rat aortic vascular smooth muscle cells and in endothelial cell tissue from anesthetized rats (42), as well as to induce a two- to threefold increase in AM mRNA levels in a variety of organs, including the lungs and intestine (41). Plasma levels reflect local hemodynamic distribution, as well as production and secretion. Thus, studying protein levels may not be an accurate measure of secretion by a given organ. By studying the time course of gene induction directly, we could localize the liver as a major site of early AM production in response to LPS, with kinetics common to those of acute-phase reactants. The particular hepatic cell type (i.e., resident histiocytic Kupffer cells, hepatocytes, or others) that is responsible for this increase remains to be elucidated, as is the relative contribution of other parenchymal tissues not examined here.

The possible role of IFN-γ in the regulation of AM gene expression is another novel aspect of this study. IFN-γ alone is nearly as potent an inducer of AM gene expression as LPS in vitro, yet in contrast to many LPS-inducible genes, where IFN-γ and LPS synergize (e.g., those for TNF-α, inducible NO· synthase, etc.) (13, 47), AM gene expression was antagonized when both IFN-γ and LPS or paclitaxel were present simultaneously. This pattern of mitigated gene expression in the presence of both IFN-γ and LPS has been reported for several other LPS-inducible genes, including those for KC, IL-1β, the type 2 TNF receptor, and the secretory leukocyte protease inhibitor (10, 16, 23). The molecular interaction that results in this antagonism is not understood. In vivo, IFN-γ has been implicated as a critical cytokine in LPS-induced toxicity (39). Mice with a targeted mutation of the IFN-γ gene (i.e., GKO mice) exhibited elevated basal AM expression that was downregulated only after LPS injection. These data support the hypothesis that IFN-γ must necessarily interact with some additional LPS-inducible inflammatory mediator to maintain AM levels in the normal mouse.

Taken collectively, our data suggest that the gene for AM may be viewed as an additional immediate-early gene produced predominantly by the liver in response to LPS. The relative contribution of AM to hypotension and septic shock remains to be elucidated by blocking its secretion or action.

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ADDENDUM IN PROOF

A very similar report has recently been published by A. Kubo, N. Mimamino, Y. Isumi, T. Katafuchi, K. Kangawa, K. Dohi, and H. Matsuo (J. Biol. Chem. **273**:16730–16738, 1998).

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Induction of Proinflammatory and Chemokine Genes by Lipopolysaccharide and Paclitaxel (Taxol™) in Murine and Human Breast Cancer Cell Lines

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Abbreviations: LPS, lipopolysaccharide; RT-PCR, reverse transcriptase-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; G-CSF, granulocyte colony stimulating factor; iNOS, inducible nitric oxide synthase; IL, Interleukin; GM-CSF, granulocyte-macrophage colony stimulating factor; TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; NO, nitric oxide; IFN, interferon; HPRT, hypoxanthine-guanine phosphoribosyl transferase; m, murine; h, human;

ABSTRACT

In murine macrophages, the anti-tumor agent, paclitaxel, induces expression of a wide variety of inflammatory and anti-inflammatory genes, and causes cytokine secretion via a signaling pathway that overlaps with that engaged by lipopolysaccharide (LPS), the endotoxic component of Gram negative bacteria. Using semi-quantitative RT-PCR for detection of gene expression, coupled with ELISA for the detection of secreted gene products, we analyzed the responsiveness of an extensive panel of cytokine and non-cytokine genes to induction by paclitaxel and LPS in the murine DA-3 breast cancer line. A subset of the genes examined (e.g., G-CSF, MIP-2, iNOS, and IL-1β, and GM-CSF) was upregulated >3-20-fold by both LPS and paclitaxel in the DA-3 cell line, while IP-10 mRNA was induced by paclitaxel but not by LPS. In the human MDA-MB-231 breast cancer cell line, LPS also increased mRNA levels for both GM-CSF and IP-10 significantly, while, paclitaxel increased IP-10 mRNA levels with delayed kinetics and failed to induce GM-CSF mRNA. Co-cultures of murine breast cancer cells and macrophages, stimulated with IFN-γ plus either paclitaxel or LPS, resulted in augmented release of nitric oxide. Since both GM-CSF and IP-10 have been implicated in tumor rejection in vivo through either indirect actions on the host immune system or by inhibiting tumor angiogenesis, our data strengthen the hypothesis that tumor cell-derived inflammatory mediators may in part underlie the anti-tumor efficacy of paclitaxel in breast cancer.

INTRODUCTION

Paclitaxel (Taxol™), originally isolated from the bark of the Pacific Yew tree, Taxus brevifolia, has potent antiproliferative activities against neoplastic cells both in vitro and in patients (1). These activities are thought to depend largely on the drug's ability to bind β-tubulin in the context of microtubules and to inhibit microtubule depolymerization, thus blocking mitosis and inducing apoptosis (2; 3). In addition to its anti-mitotic effects, paclitaxel can induce gene expression, including genes encoding transcription factors, membrane proteins, and cytokines such as IL-1β and IL-8 (4-6). Moreover, paclitaxel-induced gene expression has been shown to occur in both immune cell types and in tumor cells. In murine macrophages, paclitaxel closely mimics the effects of Gram negative LPS, inducing the expression of cytokines such as TNFα and IL-1β (6; 7), chemokines such as IP-10 (8), as well as non-cytokine genes such as adrenomedullin (9). In these cells, the intracellular signal transduction pathways induced by paclitaxel appear to be very similar to that of LPS, as paclitaxel-induced gene expression is blocked by LPS structural analog antagonists (10) and is not observed in mice that are genetically hyporesponsive to LPS (7). Furthermore, both paclitaxel and LPS activate the nuclear transcription factor NF-κB (11; 12). Less is known about the ability of paclitaxel to regulate "LPS-inducible" genes in tumor cells, although paclitaxel-induced IL-8 expression by ovarian cancer cells (13) and IL-1β expression by breast cancer cells have been demonstrated (6). Conversely, LPS has indirect anti-tumor effects on its own, and is being employed in clinical trials as an ex vivo activator of macrophages prior to their re-infusion into cancer patients in adoptive immunotherapy (14).

The structure-function relationships for paclitaxel's β-tubulin binding and LPS mimetic capacities appear to be dissociable for certain genes, e.g., TNFα and iNOS, but not for others, e.g., IL-8 (4; 15-17), and the picture is further complicated by species-specific differences (18). It has thus been hypothesized that the anti-tumor effects of paclitaxel may, in part, be mediated by gene induction; however, the relative contribution of

gene induction to the overall pharmacological properties of paclitaxel remains to be elucidated.

In the tumor bed, cytokines can be secreted by both tumor-infiltrating immune cells and by the tumor cells themselves, and can have pleiotropic effects. Having previously found an "LPS-mimetic" effect of paclitaxel on cytokine gene induction in macrophages, we were interested in the potential ability of paclitaxel and LPS to stimulate gene induction directly in breast cancer cells, as paclitaxel has shown clinical efficacy in this disease. Specifically, we chose to study a number of cytokine genes involved in leukocyte recruitment and differentiation including the colony stimulating factors (G-CSF, M-CSF, and GM-CSF), chemokines (MIP-1β and MIP-2, IP-10), and proinflammatory cytokines, such as IL-1β. GM-CSF can recruit monocytes and stimulates their differentiation into dendritic cells. Thus, GM-CSF is being clinically tested as an adjuvant for cancer immunotherapy (19), and indirect host-dependent anti-tumor effects have been postulated for other cytokines and chemokines. In the present work, we first analyzed the LPS- and paclitaxel responsiveness of a murine breast cancer cell line, DA-3 (20). Both LPS and paclitaxel led to the induction of a significant number of genes not normally considered to be products of breast cancer cells. When an analysis of two of these genes was also compared in the human breast cancer cell line, MDA-MB-231 (21), LPS and paclitaxel were found to increase mRNA expression for GM-CSF and IP-10 in a species- and stimulant-specific fashion. Co-culture of murine breast cancer cells with murine macrophages resulted in augmented release of nitric oxide ($NO^{\cdot}$) by macrophages stimulated with LPS or paclitaxel and IFN-γ. Our data support the hypothesis that the anti-tumor effects of paclitaxel may, in part, be mediated by the capacity of this agent to elicit in breast cancer cells and macrophages the expression of genes that encode proinflammatory cytokines and chemokines.

MATERIALS AND METHODS

Reagents. Phenol-water-extracted *E. coli* K235 LPS (LPS; <0.008% protein) was prepared by the method of McIntire et al. (22). Paclitaxel was kindly provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, and was stored at -70° C as a 20 mM stock solution in dimethylsulfoxide. A 1 mM stock of paclitaxel contained <0.03 endotoxin U/ml by the *Limulus* amoebocyte lysate assay.

Cell cultures. The DA-3 murine breast cancer cell line was a kind gift of Dr. D. M. Lopez (Department of Microbiology and Immunology, University of Miami School of Medicine, FL 33136, USA). MDA-MB-231, a human breast cancer cell line, was obtained from the American Type Culture Collection (Manassas, VA). After trypsin treatment, cells were washed three times with PBS and resuspended in DMEM medium supplemented with 2 mM glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin per ml, 10 mM HEPES, 0.3% sodium bicarbonate, 10% fetal calf serum, and added to six-well tissue culture plates (Falcon, Lincoln Park, NJ) at ~3.0 x 10^6 cells per well in a final volume of 2.0 ml. The plates were incubated at 37° C and 6% CO_2. After 12 h, nonadherent cells were washed off and the adherent breast cancer cells were treated with 2.0 ml of medium or medium containing the indicated substances.

In certain experiments, thioglycollate-elicited macrophages (4 x 10^6) derived from C3H/OuJ and C3H/HeJ macrophages as described previously (23) were co-cultured with DA-3 cells (3 x 10^6) in 6 well culture plates in the presence or absence of paclitaxel (35 μM) or LPS (100 ng/ml) and/or IFN-γ (5 U/ml). Supernatants were collected after 24 h treatment and nitricoxide production was assessed by measuring nitrite release into culture supernatants using the Greiss reaction (23).

Isolation and analysis of mRNA by reverse transcription (RT)-PCR. Culture supernatants were removed and the cells were solubilized and homogenized in

RNA Stat60 (Tel-Test 'B', Inc., Friendswood, TX). Total cellular RNA was extracted from *in vitro* samples according to the manufacturer's instructions and quantified by spectrophotometric analysis. Relative quantities of mRNA for the housekeeping genes, murine hypoxanthine-guanine phosphoribosyl transferase (HPRT) (24), and human β-actin (25), and for the genes of interest were determined by a coupled RT-PCR as detailed elsewhere (26). The annealing temperatures, and cycle numbers used for the analysis of genes are detailed in Table 1. The sense and antisense primers for the genes examined, as well as probes used for southern blot analysis, have been previously published (9; 23; 25; 27-29; 29). The sequences of hIP-10 primer pairs are as follows: 5'-AAGGATGGACCACACAGAGG (sense) and 5'-TGGAAGATGGGAAAGGTGAG (antisense). The sequence of the hIP-10 oligonucleotide probe is 5'-GTGGATGTTCTGACCCTTGCT. The probe sequences were chosen in conjunction with the published cDNA sequences obtained from GenBank. Amplified products were analyzed by electrophoresis followed by Southern blotting and hybridization with a nonradioactive internal oligonucleotide probe. Chemiluminescent signals were quantified using a scanning densitometer (Datacopy GS plus, Xerox Imaging Systems, Sunnyvale, CA). To determine the magnitude of change in gene expression, cDNA from a sample known to be positive for the gene analyzed were used to generate standard curves by serial 2-fold dilution of the positive control and simultaneous amplification. The signal of each band in the standard curve was plotted and subjected to linear regression analysis. The equation from this line was used to calculate the fold induction in test samples. Results were normalized for the relative quantity of mRNA by comparison to HPRT or β-actin. In each *in vitro* experiment, means are expressed relative to medium controls.

Detection of GM-CSF or IP-10 protein. Culture supernatants were analyzed for murine or human GM-CSF protein levels by ELISA kits (R&D Systems, Minneapolis, MN), according to manufacturers' recommendations. Human IP-10 was

kindly measured by a non-commercially available ELISA developed in the laboratory of Dr. Andrew Luster (Massachusetts General Hospital, Boston, MA).

Statistical analysis. Results were analyzed using Student's *t* test for comparisons between two groups.

RESULTS

Genes induced by LPS and paclitaxel in the murine breast cancer line DA-3. The murine breast cancer cell line, DA-3, has been reported to secrete GM-CSF constitutively, and to depress macrophage activation in tumor-bearing hosts (20). DA-3 cells were treated with LPS (100 ng/ml) or paclitaxel (35 μM), concentrations found to induce maximal levels of mRNA in murine macrophages. Total RNA was isolated and levels of steady-state mRNA expression were assessed by RT-PCR. Table 2 lists those genes that were not detected in RNA derived from the DA-3 cells over an 8 h timecourse, even after 40 cycles of PCR amplification, without or with treatment (data not shown). Of the genes for which basal mRNA levels could be detected, NM-23 (A) and M-CSF were only slightly induced (<3-fold) by LPS or paclitaxel (data not shown). In contrast, moderate to high increases (>3 to 30-fold) in mRNA levels were observed for G-CSF, MIP-2, iNOS, IL-1β, GM-CSF, and IP-10.

Kinetics of induction of IL-1ß, G-CSF, MIP-2, and iNOS mRNA by LPS or paclitaxel. The induction of genes induced at moderate to high levels was more extensively characterized. In DA-3 cells, the kinetics and magnitude of induction by LPS or paclitaxel of IL-1β, G-CSF, MIP-2, and iNOS was very similar (Figure 1). All four genes were induced within 2 hr of stimulation, and peak levels seen 2-6 h after stimulation. By 24 h after treatment, steady-state mRNA expression of all four genes had begun to decline, although iNOS mRNA expression remained significantly elevated. As has been observed in murine macrophages (23), the similar and often overlapping kinetic profiles of gene induction by paclitaxel and LPS suggest that they operate via a shared signal transduction pathways in the DA-3 breast cancer cell line.

Kinetics of GM-CSF and IP-10 mRNA induced by LPS or paclitaxel in murine and human breast cancer cell lines. Analysis of the kinetics of LPS- and paclitaxel-induced murine (m) GM-CSF and IP-10 gene expression in the DA-3 cell

line revealed some notable differences. The kinetics of mGM-CSF mRNA expression induced by LPS and paclitaxel in the murine DA-3 cell line were very similar, peaking by 2 h, and returning rapidly to basal levels by 24 h (Fig. 2; top left panel). The peak magnitude induced by LPS was consistently one of the highest elicited among all fourteen genes in our panel. In contrast, the expression of the IP-10 gene was induced much more strongly in DA-3 cells by paclitaxel than by LPS, resulting in a maximum induction of ~30-fold above basal levels at 24 h following stimulation with paclitaxel (Fig. 2; bottom left panel).

We also analyzed the expression of both genes in a human breast cancer cell line, MDA-MB-231, which, like the murine DA-3 line, has been shown previously to express constitutive levels of GM-CSF (21). However, LPS, but not paclitaxel, induced expression of human (h) GM-CSF mRNA in the human MDA-MB-231 cell line (Fig. 2; top right panel). In contrast, both agents strongly induced hIP-10 gene expression, peaking at 2 and 8 h following LPS stimulation and paclitaxel stimulation, respectively, with levels remaining elevated (~20-30-fold) for >24 h (Fig. 2; bottom right panel).

Dose dependencies of GM-CSF gene induction in DA-3 murine cell line. To assess the sensitivity of the DA-3 cell line to induction of mGM-CSF by LPS or paclitaxel, dose response analyses were performed (Figure 3). The DA-3 cell line was treated for 2 h, the time when mGM-CSF mRNA expression had peaked, with various concentrations of LPS or paclitaxel. RNA was isolated and GM-CSF and HPRT mRNA was detected by RT-PCR. As little as 0.01 ng/ml LPS induced a detectable increase in GM-CSF mRNA expression, while higher concentrations were necessary to induce maximal GM-CSF mRNA expression. A comparable increase in GM-CSF gene expression was induced by 5 to 35 μM paclitaxel. These dose-response relationships establish the sensitivity of the murine DA-3 cell line to be comparable to that observed in murine macrophages treated with LPS or paclitaxel (9).

LPS induced GM-CSF and IP-10 protein secretion. In addition, culture supernatants from some of the above experiments were collected and assayed for GM-CSF protein by ELISA. DA-3 cells treated for 24 h with media alone secreted constitutive levels of mGM-CSF (284 pg/ml), confirming a previous observation (20). LPS- and paclitaxel-treated DA-3 cells secreted only slightly more mGM-CSF (405 and 361 pg/ml, respectively). In contrast, the human MDA-MB-231 cell line was stimulated by LPS to secrete ~10-fold more hGM-CSF than media- or paclitaxel-treated cells (Fig. 4). Similar results were observed for the production of hIP-10, where medium- and paclitaxel-treated MDA-MB-231 cells secreted hIP-10 below the level of detection ($\leq$100 pg/ml), whereas LPS-stimulated MDA-MB-231 cells produced 4,946 $\pm$ 350 pg/ml hIP-10. Due to the low sensitivity of the murine IP-10 ELISA, we could not determine if IP-10 protein was produced by LPS- or paclitaxel-stimulated DA-3 cells.

Effect of LPS or paclitaxel on macrophage NO release in the presence of breast cancer cells. We have previously reported that LPS and paclitaxel synergize with IFN-γ to induce NO release by LPS-responsive (C3H/OuJ) macrophages, but not in LPS-unresponsive (C3H/HeJ) macrophages (23). To determine if the presence of tumor cells modulates macrophage NO production, murine breast cancer cells and macrophages were co-cultured and treated with medium only, IFN-γ (5 U/ml), LPS (100 ng/ml), paclitaxel (35 μM), or the combination of stimuli. Neither DA-3 cells nor C3H/HeJ macrophages responded to any stimuli to release NO. The failure of C3H/HeJ macrophages to release NO was not reversed by the presence of DA-3 breast cancer cells and LPS/paclitaxel and IFN-γ. However, C3H/OuJ macrophages stimulated by IFN-γ plus either either LPS or paclitaxel secreted significantly more NO than macrophages stimulated with either stimulant alone, confirming our previous observation of synergistic induction of iNOS and NO release in these macrophages (23). The concurrent presence of the DA-3 cells in the C3H/OuJ macrophage cultures resulted in a significant increased in macrophage secretion of NO in response to paclitaxel or LPS plus IFN-γ.

DISCUSSION

Nearly three decades after its initial characterization (30), paclitaxel has proven to be an effective anti-cancer drug in patients with advanced ovarian and breast cancer (31; 32). Since the pioneering studies of Horwitz and co-workers, the mechanism of paclitaxel action has been thought to center primarily around its capacity to inhibit microtubule dissociation (3). However, other microtubule binding drugs (e.g., colchicine) are not nearly as effective as paclitaxel, and despite intensive research, the mechanism by which paclitaxel is selective against tumor cells remains unknown. In recent years, we and others have shown that in murine macrophages, paclitaxel induces the expression of a number of genes and secretion of many inflammatory gene products, via a signal transduction pathway that is indistinguishable from that of LPS (7; 8). Since many of the cytokine genes induced by LPS and paclitaxel can mediate tumor regression, the possibility that this gene-inducing activity is responsible for at least some of the anti-tumor activity seen in vivo remains a viable hypothesis. We report here that both LPS and paclitaxel can induce the expression of a number of immunomodulatory genes in breast cancer cells as well.

Although expression of a subset of genes was below the limit of detection in the murine DA-3 cell line (Table 2), a number of genes were readily detected in the absence of exogenous stimulation and their expression could be upregulated by both LPS and paclitaxel (Table 2; Fig. 1). Of these, M-CSF is notable, as a majority of human breast tumors have been found to express M-CSF constitutively, leading to the proposal that its expression may be linked to invasive potential (33). IL-1β mRNA was also up-regulated by both agents. IL-1β induction by paclitaxel has been reported for human breast cancer cell lines under similar conditions (6). In a recently published study, induction of IL-1β secretion by a malignant lymphoma abrogated tumorigenicity, while IL-1β antagonists restored lethal progression (34). However, IL-1β has been found to be expressed in most invasive breast cancer tumors, where expression levels were higher in invasive than in pre-invasive or benign lesions (35). G-CSF is a cytokine that is capable of neutrophil

recruitment and maturation (36). The kinetic profiles of LPS- and paclitaxel-induced G-CSF gene expression in DA-3 cells were very similar to each other, as well as to that of IL-1β, suggesting that both genes may be regulated in a like manner by these two agents, potentially through NF-κB (11; 12). MIP-2, which was also upregulated by LPS and paclitaxel, is closely related to IL-8, a chemokine shown to be induced by paclitaxel in ovarian cancer lines (13). However, the finding that MIP-2 is overexpressed by tumor cells in a transgenic model of spontaneous breast cancer, raises the possibility that it is oncogenic (37). One important cavaet to be considered in experiments where the level of a single cytokine gene is manipulated, is that it is likely that the effect on tumor progression/regression is not what would be observed following the coordinate upregulation of a subset of genes, as is observed in Fig. 1 in response to LPS or paclitaxel.

In contrast to the similarity of induction of the aforementioned subset of genes, steady-state levels of mGM-CSF mRNA were significantly, but transiently, increased by both LPS and paclitaxel in the mouse DA-3 breast cancer cell line. However, levels of secreted mGM-CSF were only modestly increased by comparison. Whether this represents a failure to release GM-CSF into the culture supernatants or the rapid re-utilization or degradation of this cytokine by this tumor cell line is unknown. Since GM-CSF levels have been reported to be increased by post-transcriptional stabilization of mRNA (38), differences in release of protein by LPS vs. paclitaxel might be due to divergent effects of these agents on signaling pathways that influence mRNA stability; however, this seems unlikely since the peak levels and kinetics of mGM-CSF steady-state mRNA induced in the DA-3 cell line were not grossly disparate. However, in macrophages, colchicine, a microtubule-binding agent, prevents LPS-induced GM-CSF secretion (39), and both colchicine and paclitaxel increase intracellular levels of LPS-induced pro-IL-1β without increasing IL-1β secretion (40). Thus, the failure to detect secreted GM-CSF in the face of strong paclitaxel-induced mGM-CSF mRNA expression (or NO release in the face of strong iNOS gene expression) may be the result of paclitaxel's well-documented ability to

result in microtubule hyperstabilization, that in turn, affects secretion adversely. It is also possible that intracellular cytokines accrue in paclitaxel-treated breast cancer cells and are released locally only when the cell undergoes necrosis secondary to paclitaxel-induced apoptosis or when microtubule function returns when paclitaxel is suspended between intervals of therapy.

To extend these studies to a model closer to human disease, we compared the effects of LPS and paclitaxel on hGM-CSF mRNA and protein levels in MDA-MB-231, a human breast cancer line (21). In contrast to the murine cell line, the response to LPS and paclitaxel diverged in the MDA-MB-231 cell line, with only LPS causing significant increases in both GM-CSF mRNA and protein levels. These results cannot be due to an inherent defect in the ability of paclitaxel to induce gene expression in MDA-MB-231 cells, as the same treatment regimen markedly increased levels of hIP-10 mRNA.

The role of GM-CSF in tumorogenicity is controversial. In DA-3 tumor-bearing mice, secretion of GM-CSF by tumor cells has been reported to be partly responsible for the immunosuppressed state of the tumor-bearing mice, due to the recruitment of suppressor cells that hinder a specific anti-tumor immune response (20; 41). In contrast, tumors engineered to produce GM-CSF are being used as cancer vaccines and GM-CSF itself is being employed as an adjuvant to immunotherapy (19; 42), in spite of the fact that it has been known for some time that tumor cells secrete GM-CSF (43), and that in head and neck cancers, GM-CSF secretion correlates with a worse prognosis (44). In vitro, GM-CSF-induced expansion of bone marrow-derived macrophages has been shown to result in macrophages with a greater potential for tumor cytotoxicity (39). In addition, GM-CSF can diminish tumor viability by upregulating the enzymatic activity by which tumor infiltrating macrophages process angiostatin, thus inhibiting neo-angiogenesis at the tumor site (45). Thus, whether the effects of GM-CSF are favorable or not to the tumor is likely to depend on the local microenvironment.

We have previously shown that both LPS and paclitaxel can induce IP-10 mRNA in macrophages (8). The data presented herein are the first to demonstrate that breast cancer cells can also express IP-10 and that the anti-cancer drug paclitaxel can increase IP-10 gene expression in both the murine and human breast cancer cell lines. These findings are important in light of the ability of IP-10 to recruit T cells, neutrophils, and macrophages to the tumor bed and to induce T cell-dependent tumor regression (46). Furthermore, IP-10 is a potent inhibitor of angiogenesis in vivo (47). Thus, while IP-10 may promote tumor invasiveness by increasing tumor cell motility (48), increased expression levels might ultimately be detrimental to the tumor via anti-angiogenic mechanisms.

Our data indicate that breast cancer cells express a number of immunomodulatory genes whose mRNA levels can be affected by both LPS and paclitaxel. At this time, the mechanism by which LPS and paclitaxel interact with breast cancer cells to elicit the intracellular signals required for induction of this array of genes is unknown. However, Western analysis of the DA-3 cell line indicates that it lacks two major LPS "receptors," CD14 and Mac-1, but expresses mRNA for Toll-like receptors (TLR) 2 and 4 (data not shown), which have been recently implicated as LPS signal tranducing molecules (49). These data are consistent with the hypothesis that the ability of paclitaxel to cause "LPS-like" gene induction in cancer cells may contribute to the anti-tumor efficacy of paclitaxel, in contrast to other microtubule binding agents that lack LPS-mimetic activity. Moreover, the anti-tumor efficacy might be dependent upon cell interactions or anti-angiogenic mechanisms that require the host and would, thus, not be observed in vitro. This is supported by the data in Fig. 5 that demonstrate that the interaction between breast cancer cells and macrophages enables a significantly enhanced macrophage response to LPS or paclitaxel, suggesting the possibility that signals produced by the tumor cells in response to LPS or paclitaxel may contribute significantly to macrophage activation for anti-tumor activity. The capacity of the murine DA-3 cells to respond to LPS or paclitaxel with the

induction of such an array of inflammatory genes may provide a model to investigate the ability to impact upon this progression by cytokine or cytokine-inducing therapies.

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FIGURE LEGENDS

Figure 1. IL-1ß, G-CSF, MIP-2, and iNOS mRNA are induced in the DA-3 murine breast cancer cell line by both paclitaxel and LPS. Cells were cultured for the indicated time with medium, 100 ng/ml LPS, or 35 μM paclitaxel, and levels of mRNA for IL-1β, G-CSF, MIP-2 or iNOS were quantified by RT-PCR. Data represent the arithmetic mean ± s.e.m. from 4 independent experiments.

Figure 2. Induction of GM-CSF and IP-10 mRNA in DA-3 (murine) and MDA-MB-231 (human) breast cancer cell lines. Cell lines were cultured for the indicated time with medium, 100 ng/ml LPS, or 35 μM paclitaxel, and the kinetics of GM-CSF and IP-10 mRNA levels were quantified by RT-PCR. Data represent the arithmetic mean ± s.e.m. from 5 independent experiments.

Figure 3. Dose-dependent induction of GM-CSF mRNA in murine DA-3 cell line. The DA-3 breast cancer cells were cultured for 2 h with medium or with the indicated concentrations of LPS or paclitaxel. Data represent the arithmetic mean ± s.e.m. from 3 independent experiments.

Figure 4. GM-CSF protein secretion induced by LPS or paclitaxel in the human MDA-MB-231 line. Culture supernatants following treatment with 100 ng/ml LPS or 35 μM paclitaxel were collected at the times indicated and assayed for GM-CSF protein content by ELISA. Data are representative of 3 independent experiments with similar results.

Figure 5. Paclitaxel or LPS plus IFN-γ induce augmented macrophage NO release in the presence of breast cancer cells. C3H/OuJ or C3H/HeJ macrophages, DA-3 murine breast cancer cells, or co-culture of C3H/OuJ and DA-3 or C3H/HeJ and DA-3, were stimulated with medium alone, 100 ng/ml LPS, 35 μM paclitaxel, 5 U/ml IFN-γ, or IFN-γ plus either LPS or paclitaxel. After 24 h, culture

supernatants were harvested for nitrite levels. Values are the arithmetic means ± s.e.m. obtained from four separate experiments. The asterisk indicates a significant difference in NO release stimulated by paclitaxel or LPS and IFN-γ in the absence vs. present of DA-3 cells.

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Table 1. Conditions for gene expression analysis by reverse transcriptase polymerase chain reaction (RT-PCR)

Species	Gene	Annealing Temp [°C]	Cycles amplified[a]
Murine (m)	GM-CSF	54	28
	G-CSF	57	26
	NM 23 (A)	50	25
	M-CSF	58	28
	IP-10	55	31
	MIP-1β	65	40
	MIP-2	65	33
	iNOS	65	32
	IL-1β	54	32
	IL-12 p40	58	40
	MCP-5	55	40
	c-kit	53	40
	AM	55	40
	JE/MCP-1	65	40
	HPRT	54	24
Human (h)	GM-CSF	56	27
	IP-10	56	33
	β-actin	53	24

[a] The number of cycles used for the detection of each gene under nonsaturating conditions.

Table 2. LPS- or paclitaxel-induced mRNA expression in mouse DA-3 breast cancer cells

Level of mRNA induction by LPS or paclitaxel		
Not Detected Not Induced[a]	**Low Induction (<3-fold)**	**Moderate to High induction (>3-fold)**
IL-12 p40	NM 23 (A) (4)[b]	G-CSF (4)
MIP-1β	M-CSF (2)	MIP-2 (2)
MCP-5		iNOS (4)
c-kit		IL-1β (4)
AM		GM-CSF (5)
JE/MCP-1		IP-10 (4)

[a]mRNA expression was undetectable with any treatment regimen after 40 cycles of PCR.

[b]Numbers in parentheses indicate the number of independent experiments.

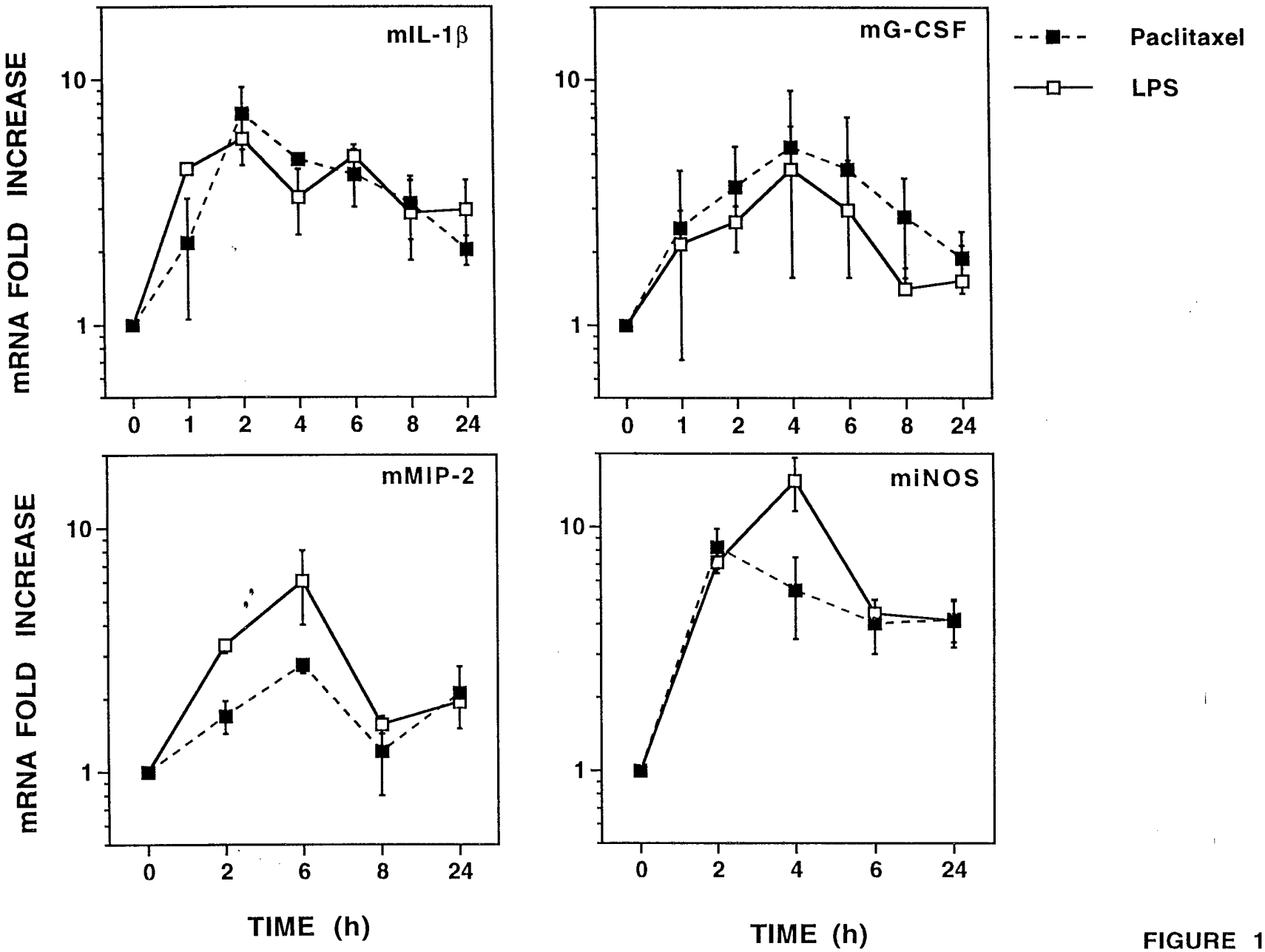
mIL-1β
mG-CSF
mMIP-2
miNOS
Paclitaxel
LPS
mRNA FOLD INCREASE
mRNA FOLD INCREASE
10
1
0 1 2 4 6 8 24
0 2 6 8 24
0 2 4 6 24
TIME (h)
TIME (h)

FIGURE 1

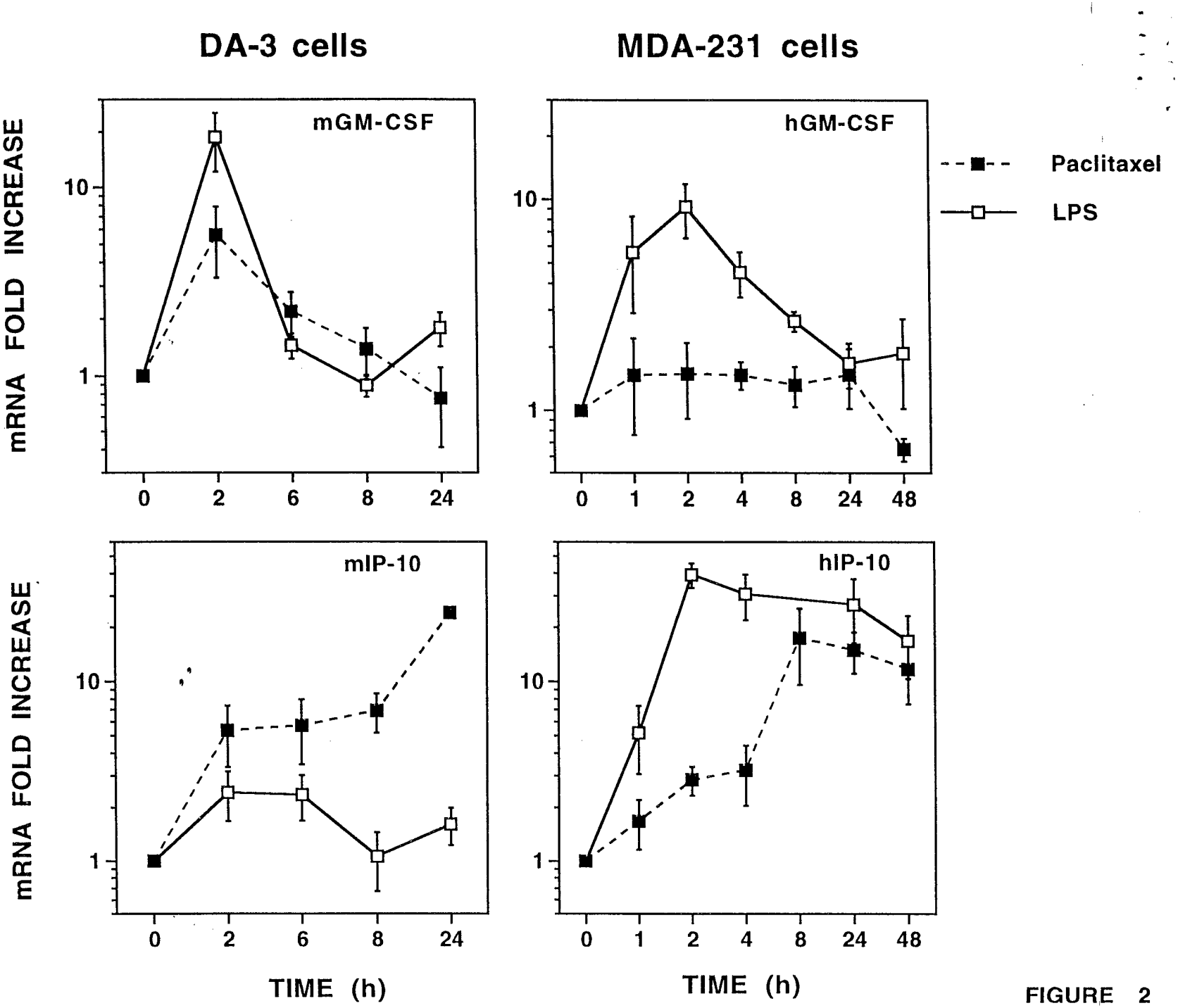
DA-3 cells
MDA-231 cells
mGM-CSF
hGM-CSF
mIP-10
hIP-10
mRNA FOLD INCREASE
mRNA FOLD INCREASE
10
1
0 2 6 8 24
0 1 2 4 8 24 48
TIME (h)
TIME (h)
Paclitaxel
LPS

FIGURE 2

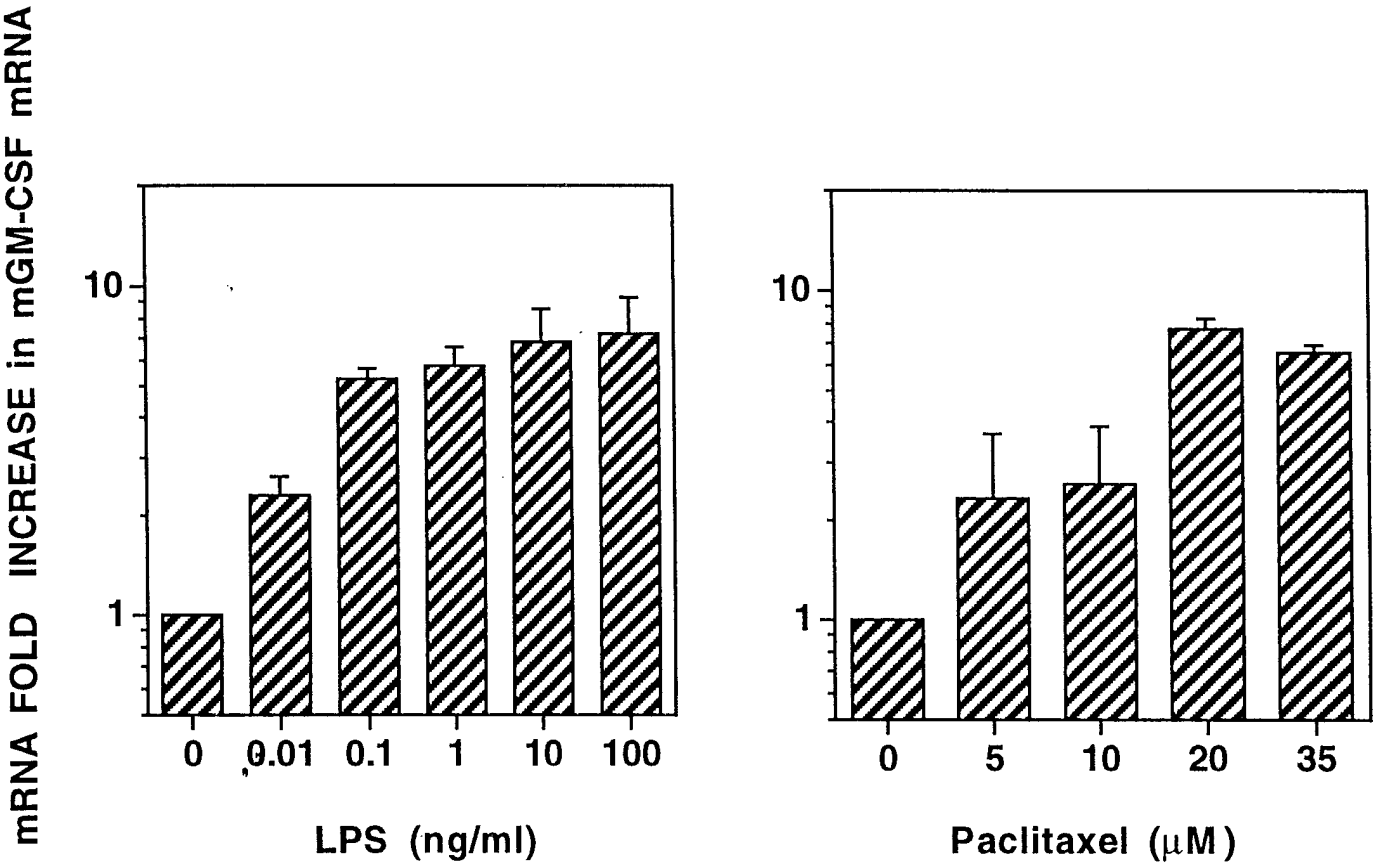
mRNA FOLD INCREASE in mGM-CSF mRNA
10
1
0
0.01
0.1
1
10
100
LPS (ng/ml)
10
1
0
5
10
20
35
Paclitaxel (μM)

FIGURE 3

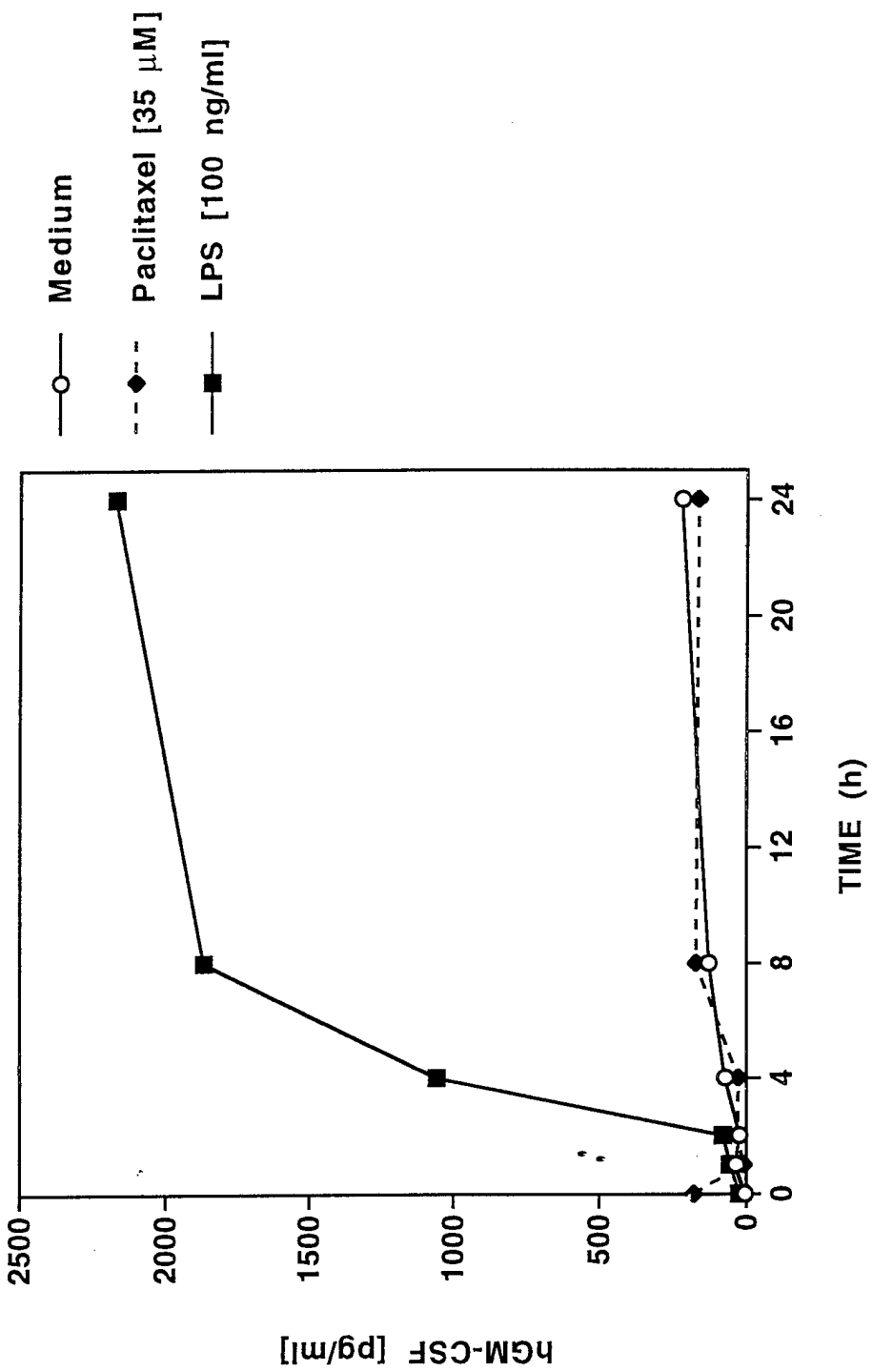
Medium
Paclitaxel [35 μM]
LPS [100 ng/ml]
hGM-CSF [pg/ml]
2500
2000
1500
1000
500
0
0
4
8
12
16
20
24
TIME (h)

FIGURE 4

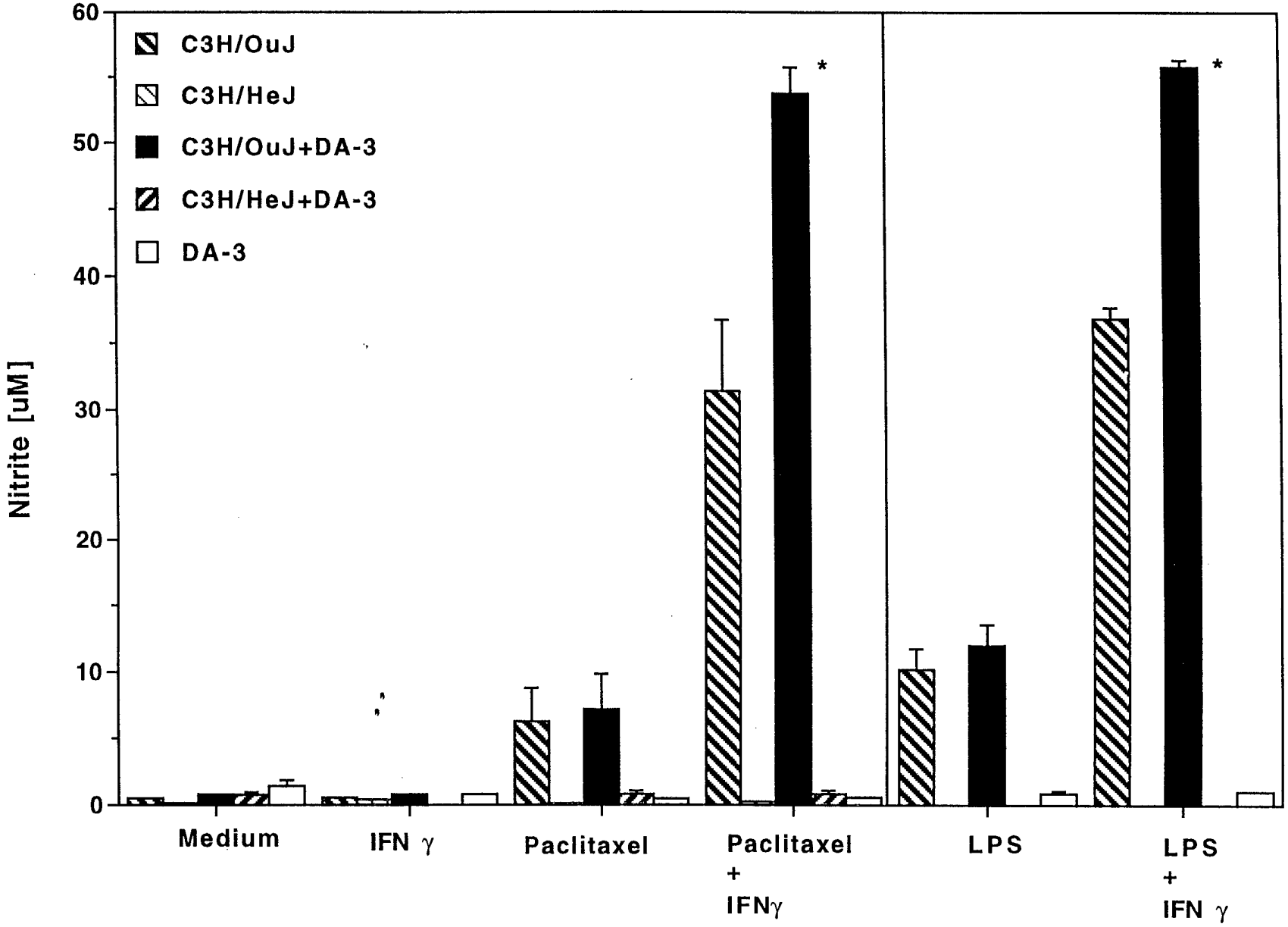
C3H/OuJ
C3H/HeJ
C3H/OuJ+DA-3
C3H/HeJ+DA-3
DA-3
Nitrite [uM]
60
50
40
30
20
10
0
*
*
Medium
IFN γ
Paclitaxel
Paclitaxel + IFNγ
LPS
LPS + IFN γ

FIGURE 5

TENTATIVE FINAL REPORT

FEDERAL CASH TRANSACTIONS REPORT

(See instructions on the back. If report is for more than one grant or assistance agreement, attach completed Standard Form 272-A.)

Approved by the Office of Management and Budget, No. 80-RO182

1. Federal sponsoring agency and organizational element to which this report is submitted

U.S. Army Medical Research Acquisition Activity

2. RECIPIENT ORGANIZATION

Name: HENRY M. JACKSON FOUNDATION FOR THE ADVANCEMENT OF MILITARY MEDICINE

Number and Street: 1401 ROCKVILLE PIKE, SUITE 600

City State and Zip Code: ROCKVILLE, MD. 20852

3. FEDERAL EMPLOYER IDENTIFICATION NO.> 52-1317896

4. Federal grant or other identification number: **DAMD 17-96-1-6258**

5. Recipient's account number or identifying number: **600-06805000-073**

6. Letter of credit number: **0**

7. Last payment voucher number: **8**

Give total number for this period

8. Payment Vouchers credited to your account:

9. Treasury checks received (whether or not deposited): **0**

10. PERIOD COVERED BY THIS REPORT

FROM *(month, day, year)*: **4/01/99**

TO *(month, day, year)*: **6/23/99**

11. STATUS OF FEDERAL CASH *(See specific instructions on the back.)*		
a.	Cash on hand beginning of reporting period	$ 11,542.13
b.	Letter of credit withdrawals	0.00
c.	Treasury check payments	0.00
d.	Total receipts (Sum of lines b and c)	0.00
e.	Total cash available (Sum of lines a and d)	11,542.13
f.	Gross disbursements	9,855.21
g.	Federal share of program income	0.00
h.	Net disbursements (line f minus line g)	9,855.21
i.	Adjustments of prior periods	0.00
j.	Cash on hand at end of period	$ 1,686.92

12. THE AMOUNT SHOWN ON LINE 11J ABOVE, REPRESENTS CASH REQUIREMENTS FOR THE ENSUING DAYS	13. OTHER INFORMATION	
a.	Interest income	$ See Remarks
b.	Advances to subgrantees or subcontractors	$ 0.00

14. REMARKS *(Attach additional sheets of plain paper if more space is required.)*

Interest will be remitted to the Department of Health and Human Services with 9-30-99 Quarterly reports.

15. CERTIFICATION

I certify to the best of my knowledge and belief that this report is true in all respects and that all disbursements have been made for the purpose and conditions of the grant or agreement

AUTHORIZED CERTIFYING OFFICIAL

SIGNATURE: Laraine Peng

DATE REPORT SUBMITTED: 7/22/99

TYPED OR PRINTED NAME AND TITLE: LARAINE PENG, FINANCE MANAGER

TELEPHONE *(Area Code, Number, Extension)*: (301)424-0800

THIS SPACE FOR AGENCY USE

This is a tentative final report. Once the outstanding encumbrances are cleared the final report with the correct figures will be sent.

272-102

*GPO: 1979 0-281-187 P.O. 5073

STANDARD FORM 272 (7-76)

Prescribed by Office of Management and Budget

Cir. No. A-110